PhD Thesis

Effects of Environmental Pollutant Heavy Metals on the Electric Activity of the Somatosensory System in Rats in Acute Application

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The Applicant's Relevant Publications

I. *Pecze L.*, Papp A.: Parallel changes of the spontaneous and stimulus-evoked cortical activity elicited by acute treatment with inorganic mercury in rats. *Centr. Eur. J. Occup. Environ. Med.* 8;126-130 (2002).

II. *Pecze L.*, Vezér T., Papp A.: Effects of acutely administered heavy metals on the evoked activity recorded from the cortical and thalamic somatosensory center in rats. *Fiziologia (Timisoara)* 12;34-38 (2002).

III. *Pecze L.*, Papp A., Nagymajtényi L.: Simultaneous changes of the spontaneous and stimulus-evoked cortical activity in rats acutely treated with mercuric chloride. *Neurotox. Teratol.*, In press, accepted 17 July 2003. Impact factor: 1.672

IV. *Pecze L.*, Papp A., Nagymajtényi L.: Acute effects of lead and mercury on the central and peripheral nervous system in rats pretreated with alcohol. *Centr. Eur. J. Occup. Environ. Med.*, In press, accepted 1 December 2003.

V. *Pecze L.*, Papp A., Nagymajtényi L.: Changes in the spontaneous and stimulus-evoked activity in the somatosensory cortex of rats on acute manganese administration *Tox. Letters,* In press, accepted 17 December 2003. Impact factor: 2.242

VI. *Pecze L.*, Papp A., Nagymajtényi L., Dési I.: Effect of acute administration of certain heavy metals and their combinations on the spontaneous and evoked cortical activity in rats. *Environ. Toxicol. Pharmacol.*, Accepted 16 January 2004. Impact factor: 1.271

VII. *Pecze L.*, Papp A., Nagymajtényi L.: The effect of acutely administered heavy metals on the somatosensory activity in rats. In: Galbács Z. (Ed.) *Proceedings of the 8th Symposium on analytical and environmental problems*. SZAB, Szeged, 2001. pp. 99-103.

VIII. *Pecze L.*, Papp A.: Acute oral exposure of rats to manganese: effects on the cortical electrical activity. In: Galbács Z. (Ed.) *Proceedings of the 9th Symposium on analytical and environmental problems*. SZAB, Szeged, 2002. pp. 46-49.

IX. *Pecze L.*, Papp A.: Neurotoxicity of lead and mercury in acute exposure. *Proceedings of the VIIth International Symposium Interdisciplinary Regional Research*, Hunedoara, Romania, 2003. pp. 89-94.

X. *Pecze L.*, Papp A.: Simultaneos changes of the spontaneous and stimulus-evoked cortical activity in rats acutely treated with mercury. In: Galbács Z. (Ed.) *Proceedings of the 10th Symposium on analytical and environmental problems*. SZAB, Szeged, 2003. pp. 106-110.

Abstracts:

XI. *Pecze L.*, Papp A., Nagymajtényi L.: Dissimilar effects of various organophosphates on the cortical and subcortical evoked activity in rats. *Neurobiology* (Budapest) 9;243-244 (2002).

XII. *Pecze L.*, Papp A., Nagymajtényi L.: Simultaneous changes of spontaneous and stimulus-evoked cortical activity in rats acutely treated with heavy metals. *Neurobiology* (Budapest) 9;349-350 (2002).

XIII. *Pecze L.*, Papp A., Nagymajtényi L.: The Effect of Acutely Administered Heavy Metals on the Electrical Activity of the Somatosensory Cortex in Rats. *Tox. Letters* 135 Suppl. 1;126 (2002). Impact factor: 2.242

XIV. *Pecze L.*, Papp A.: The effect of the acutely administered heavy metals manganese and lead on the electrical activity of the somatosensory cortex in rats. *Clinical Neuroscience (Ideggyógyászati Szemle)* 56;68-69 (2003).

Abstract

Exogenous substances affecting the nervous system are of especial interest because the functional integrity of the latter has major influence both on the individuals' life conditions and the prosperity of the society. Animal experimentation is inevitable in investigating toxic mechanisms and in developing safety information.

In this study, experiments performed with acute intraperitoneal treatment with the environmental neurotoxicant heavy metals lead, mercury and manganese, and their combinations with each other and with alcohol, are described. Male Wistar rats were anaesthetised with urethane, the head was fixed in a stereotaxic frame and the left hemisphere was exposed. Weak electric shocks to the whiskers served as stimuli. Spontaneous and stimulus-evoked activity was recorded from the primary projection area of the whiskers and the tail. After an hour of control recording, one of the following was given to the rat ip.: 1000 mg/kg Pb^{2+}, 7.0 mg/kg Hg^{2+}, 50 mg/kg Mn^{2+}; 500 mg/kg Pb^{2+}, 3.5 mg/kg Hg^{2+}, 25 mg/kg Mn^{2+}; 500 mg/kg Pb^{2+} + 25 mg/kg Mn^{2+}, or 500 mg/kg Pb^{2+} + 3.5 mg/kg Hg^{2+}. Other animals pre-treated with alcohol (5% in the drinking water for 3 to 4 weeks) and acutely treated with the heavy metals lead and mercury (500 mg/kg Pb^{2+}, 25 mg/kg Mn^{2+} and 3.5 mg/kg Hg^{2+}, ip.).

We investigated the changes on the latency and the amplitude of evoked potential, on the conduction velocity and amplitude of the peripheral nerve, and on the spontaneous activity after the metals administration. The general effect was slowing of the spontaneous cortical activity, increased amplitude and latency of the cortical evoked potentials, and decreased nerve action potential amplitude and conduction velocity in the tail. The effects was significant the most times with high dose Hg, followed by Mn and Pb. Potentiation in the metal-metal and metal-alcohol combinations were observed in the effect on stimulus-evoked activity forms.

The observed alterations of the spontaneous and stimulus-evoked cortical activity probably reflected a specific action of the heavy metals on central nervous mechanisms. Details of the toxic mechanism of the substances used in this study are not yet clear. The results indicate all the same that combined exposure of humans to the three heavy metals lead, mercury and manganese, and alcohol may have unexpectedly severe consequences.

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1. Introduction

1.1. Heavy metals in human culture and in human environment

Heavy metals have been known, and used in a variety of ways, since times well before Christ. The use of certain metals was typical for some ancient or even prehistoric cultures to that extent that the definition of archaeological ages, like copper, bronze or iron age, was based on that. The old Egyptians knew, beyond the noble metals gold and silver, also copper, lead and tin. The use of mercury in refining of gold is likewise an ancient method, used even today with considerable environmental burden and human exposure.

Before influenced by human activity, the natural environment was low in heavy metals. Most of them existed in form of low-solubility compounds in rocks and soils (generated by weathering of the rocks) and those present in elemental form has also minimal solubility. Thus, the bioavailability of metals was generally poor. This, on one hand, may have resulted in insufficient supply with essential heavy metals (e.g., iron and manganese) but meant on the other hand that the presence of toxic metals was negligible. Technical culture altered this situation dramatically by mining ores, smelting metals and forming them to commodities, and using natural metal compounds in various ways. This way, human environment (formed by humans for humans) became metal-laden well before the development of large-scale industrial activity.

The Romans added "lead sugar" (lead acetate) to wine to improve its taste, and mercury was used in those times as a salve to alleviate teething pain in infants. One historian/toxicologist contends that the Fall of the Roman Empire was hastened by the chronic lead poisoning experienced by the ruling classes who had water conducted through lead plumbing and drank wine from goblets which had lead alloy composition (Eaton and Robertson, 1994).

The toxicity of these metals has also been documented throughout history: Greek and Roman physicians diagnosed symptoms of acute lead poisoning long before toxicology became a science. Today, much more is known about the health effects of heavy metals.

The industrial revolution, which began about 1700 in Britain and subsequently spread to the rest of the world, brought in steam engines and the exploitation of coal. It was also responsible for the development of modern iron and steel industry and later the production of alloys. Since the industrial revolution, the production of heavy metals such as lead, copper,

and mercury has increased exponentially. Between 1850 and 1990, an almost 10-fold increase was recorded, with emissions rising in tandem (Nriagu, 1996).

Despite abundant evidence of their deleterious health effects, exposure to heavy metals continues and may increase in the absence of concerted policy actions. Mercury is still extensively used in gold mining in many parts of Latin America. Arsenic, along with copper and chromium compounds, is a common ingredient in wood preservatives. The use of lead additive in petrol is still practised in a number of countries, and in some of those where it was banned, was replaced by a manganese compound. Increased use of coal may increase metal exposures because coal ash contains a number of toxic metals (lead, zinc, cadmium etc). For countries which continue to rely on high-ash coal as a primary energy source (e.g. India or China) the health implications are ominous (Simon, 1995).

The consequence of use of heavy metals in industrial products is the increasing level of heavy metals in waste. Lead-acid batteries, plastics, fishing utensils, cathode ray tubes of TVs and displays, ceramics and many other minor products containing lead end up at dumpsites. Dental amalgam, measuring and control devices including thermometers, batteries, luminescent tubes and lamps etc. are important sources for mercury in waste. Spent dry cells contribute to the manganese (and cadmium) content of solid household waste.

In spite of the above-mentioned facts, pollution of the environment and its consequences were long time disregarded. In developed countries, regulations on emission of pollutants and ecotoxicants are quite strict. However, if the implementation of the regulations is inefficient, manufacturing and use of heavy metals and derivatives can result in major environmental and occupational hygienic problems. Consequently, investigation of the biological effects and roles of heavy metals can contribute to better environmental and occupational health prevention and to protection of the global environment.

1.2. General aspects of the toxicity of heavy metals

Virtually all metals can produce toxicity when ingested in sufficient quantities. Several of them are, however, especially important because either they are so pervasive, or produce toxicity at such low concentrations. Concerning environmental hygiene, the most important heavy metals are lead, mercury, manganese, cadmium and arsenic.

Any toxicant can exert its harmful action only after being absorbed and transported to the site of action within the organism. Heavy metals, when present in the air as metal fumes or respirable dust particles, can be efficiently absorbed from the airways and can have strong local effects. Oral exposure is possible via food or drinking water. From the skin, organo-

metal compounds (being typically lipophilic) are well absorbed but water-soluble inorganic metal compounds usually not. Water or fat solubility is also important in determining the distribution of any toxic substance within the organism. Water soluble compounds of lead, for example, are found (among other places) in the red blood cells while fat soluble ones are concentrated in the central nervous system (CNS). The distribution of a toxic substance determines its concentration at a particular tissue and therefore the number and type of cells exposed to high concentrations of it.

There are a few common mechanisms of toxicity of heavy metals. One of these is forming complexes with organic compounds of the body (which here act as ligands). The most common groups involved in ligand formation are polar moieties containing oxygen, sulphur and nitrogen. Heavy metals especially tend to bind to sulfhydryl (-SH) groups in enzymes and other proteins. (Lead, cadmium and mercury have such a propensity to bind to sulphur that these are in fact found in the earth's crust as sulphides.) Binding to these groups, metals alter the structure of proteins (denaturation) which in turn inactivates important enzyme systems (Bondy, 1986; Daggett et al., 1997; Markovac and Goldstein, 1988; Planas-Bohne and Montserrat, 1992). Finally, this results in malfunction or death of the affected cells.

Calcium is one of the most important messengers in intracellular signal transduction. Intracellular calcium level is regulated by a variety of mechanisms including sequestration and storage in organelles, and selective voltage- or ligand-gated channels in the cell membrane and in intracellular membranes. Voltage-gated calcium channels, e.g., are blocked by a variety of divalent and trivalent metal cations including manganese (Pumain et al., 1987) lead, mercury and zinc (Büsselberg, 1995). The calcium-dependent release of transmitters is another signalling process affected by certain heavy metals (Braga et al., 1999a; Takeda et al., 2003).

Heavy metals induce metallothionein (MT) proteins. Metallothioneins regulate blood levels of essential heavy metals, detoxify mercury and other heavy metals, and assist in neuronal development. There are four primary types of MT protein: MT-I and MT-II are found in cells throughout the body, with MT-Ill restricted primarily to the brain and MT-IV to squamous epithelial cells in the intestines. The roles of the various MT proteins and isoforms are the subject of intensive research (Theocharis et al., 2003).

1.3. The toxicity of mercury

This metal is found in three main chemical forms: metallic mercury (also known as elemental mercury, Hg°), inorganic mercury and organic mercury. Because mercury occurs naturally in the unspoilt environment, everyone is exposed to very low levels of mercury in air, water, and food. Human activities emitting mercury in the environment began in antique times but large-scale Hg pollution (by mining, burning of fossil fuels, chemical industrial applications) started with the industrial age. Metallic mercury is used in some household products and in various industrial items. Mercury in small devices like thermometers generally does not pose a risk unless the device is damaged or broken. Bulk amounts of liquid mercury, however, as e.g. in chloralkali plants, cause occupational exposure and environmental burden. A frequent source of exposure to metallic mercury for the general population is mercury released from dental amalgam fillings (WHO, 1991)

The absorption of elemental mercury from the skin and the intestines is low but mercury vapours are absorbed from the lungs at ca. 80%. The absorbed Hg° is lipophilic enough to cross barriers and is readily oxidized to Hg^{2+}. Does this happen in the brain, Hg^{2+} will be trapped within the CNS.

Inorganic mercury salts used to have a number of applications as fungicides (in wood preservatives, seed dressings, antifungal paints etc.) and medicinal products. Today, only some topical antiseptics and drug preservatives remained in use. Seed dressings containing any form of mercury are out of use by now after several episodes of mass poisoning. In case of human contact, Hg^{+} and Hg^{2+} salts are well absorbed from the intestines but much less from the skin. The primary target organs of inorganic mercury are the kidneys.

From environmental hygienic point of view, the organic mercury derivative existing in highest amounts and posing the most general exposure risk is methyl mercury, produced by microorganisms (mainly in water) from Hg emitted in the environment in other chemical forms such as industrial sewage or spent silver oxide button cells. As evidenced by the infamous Minamata case, methyl (and to a lesser extent, dimethyl) mercury, being lipophilic, accumulates in living creatures and is bioamplified along the food chain. As a result, people may be exposed to high levels of mercury in organic form if their diet is high in fish from mercury-contaminated waters. Phenyl mercury is added to water-based dispersion paints to avoid microbial decay. Organic mercury is neurotoxic, first of all for the developing, but also in the adult CNS (ATSDR, 1999b).

The nervous system in general is one of the main targets of mercury within the human organism. The neurotoxic effects are variable from neuromuscular disorders (Kark, 1994) to

abnormalities of the higher nervous functions (Zavariz and Glina, 1992). Acute inhalation of elemental mercury vapour causes acute pneumonitis and bronchiolitis. In Hg° vapour poisoning, respiratory symptoms, along with headache, nausea, general malaise, constitute the initial phase. These are followed by neurological effects including delirium, tremor, and coma developing within 24 hours (Jaeger et al., 1979). In long-term occupational exposure to inorganic mercury, alterations of the cortical electrical activity have been reported. Slowed EEG was found (in chloralkali workers; Piikivi and Tolonen, 1989), as well as amplitude increase of the somatosensory evoked potential (Lille et al., 1988) and delayed waves in the brainstem auditory evoked potential (Discalzi et al., 1993; both in several jobs involving Hg). Fine tremor of hand motions, more severe in the dominant hand, is typically seen after years of exposure to elemental or inorganic mercury (Friberg and Nordberg, 1972). Peripheral axonal neuropathy is another known consequence of mercury exposure (Singer et al., 1987).

In animal experiments, the neurotoxic effect of mercury has been described at several levels of organisation. At the molecular level, mercury - in both inorganic and organic form - was found to affect various ion channels in the peripheral and central nervous system, including both voltage-gated ones participating in the action potential mechanism and ligand-gated channels with postsynaptic or extrasynaptic localization. (Sirois and Atchison, 1996). As mentioned above, calcium homeostasis, an important factor of normal neuronal function, is prone to disturbances by heavy metals. Interfering with Ca uptake of the endoplasmic reticulum (Freitas et al., 1996) and inhibiting Ca-influx in the presynaptic endings (Denny and Atchison, 1996), Hg^{2+} can decrease the stimulus-dependent and increase the spontaneous release of transmitters. Elevated level of the transmitters noradrenaline (Gasso et al., 2000), and dopamine and serotonin (Lamm and Pratt, 1985) were in fact observed in rats treated with Hg^{2+}. Ligand binding of muscarinic receptors was inhibited by mercury compounds in rat cortical neuronal membranes in vitro (Castoldi, 1996). At the level of organs, damage to motor axons by mercuric chloride, a possible analogue of human peripheral neuropathy, was described (Pamphlett and Coote, 1998).

In earlier studies of the Neurotoxicological Laboratory of the Department of Public Health, it was found that subchronic mercury-treatment dose- and time-dependently changed spontaneous and evoked bioelectric activity in anaesthetised rats (Schulz et al., 1997).

1.4. The toxicity of lead

Unlike mercury, lead is not emitted into the environment by any natural phenomenon. As, however, it has been one of the metals to be used by man for thousands of years, lead has

become a ubiquitous environmental pollutant. Most of the environmental lead originating from human activity ends up bound to soil particles. Lead levels in natural bodies of water are usually very low (ATSDR, 1999a). The absorption of lead from the upper soil by cultivated plants, resulting finally in food-borne population exposure, depends on several factors including soil pH (potentially decreased by acid rain, also due to man-made pollution). Occasionally, lead from other sources can also appear in food items (in Hungary, red lead oxide was added to ground red paprika to improve its colour. Sold illegally, it caused mass intoxication; Kákosy et al., 1995,1996).

Lead has been, and still is, used in large amounts for a number of purposes. Metal and inorganic lead occurs in batteries, piping, paints, solders etc. Tetraethyl lead as car fuel additive is still in use in a number of countries. Before the use of unleaded petrol became general in developed countries, car fuel was by far the single largest source of lead exposure in urban areas and along main roads. In the mid-90's, approximately 90 percent of all lead emissions into the atmosphere were due to the use of leaded petrol (Lovei, 1996). Tetraethyl lead is oxidized during combustion in car engines so that lead is emitted in the exhaust gases in inorganic forms (mostly fine particles of PbO_2). In Hungary, the ban of leaded petrol in 1999 brought about a tenfold decrease in environmental lead levels. Besides car fuel, human exposure to lead results from using lead-based paints, having lead pipes in water supply systems; and exposure to industrial sources. (In the southern outskirts of Budapest, local emission from lead reclamation at the Metallokémia industrial plant since 1910 caused high lead levels in the soil, resulting in dust-borne and indirect food-borne exposure of the local population. The measured lead levels were as high as to justify the shutdown of the plant in 1990.) Additional sources of lead include soldered seams in food cans, ceramic glazes and cosmetics (Silbergeld, 1995). Besides lead metallurgy itself, occupational exposure to lead used to be common in various occupations like typesetting, painting and car maintenance. With the development of technology, few of these remained but cleaning or demolition of steel structures treated originally with lead-based anti-corrosion paint, or refurbishment of old buildings, can be a source of exposure and environmental burden even today.

There are several targets of lead in the human organism. The nervous system is among the major targets, together with the haemopoetic, gastrointestinal and endocrine systems (Thould, 1961). Lead, absorbed in any form, is accumulated in the central nervous system, first of all in the cortex and hippocampus (Grandjean, 1978).

In acute exposure due to intake of inorganic lead, encephalopathy is the most serious toxic consequence, characterised by increased intracranial pressure and brain oedema

(Feldman, 1982). Lead produces encephalopathy at blood lead levels of 100-120 μg/dl in adults and 80-100 μg/dl in children (Chrisholm, 1965). Exposure to low levels of Pb has been associated with behavioural abnormalities, learning impairment, decreased hearing, and impaired cognitive functions in humans and in experimental animals (Shannon and Graef 1992; Ruff et al.,1996). For young children, lead is a special hazard. Several studies have shown that lead exposures can significantly reduce the IQ of school-aged children; some estimates suggest that every 10 μg/dl increase in blood lead level is associated with a 1-5 point decrease in the IQ of exposed children (Goyer, 1996). IQ differences in Hungarian schoolchildren, attributable to air-borne lead, were described by Füzesi (1997). In lead-exposed children, abnormalities of cortical spontaneous and evoked activity have been found (Otto et al., 1982; Winneke et al., 1994). In adults occupationally exposed to lead, alterations of various forms of central and peripheral evoked activity, like sensory evoked potentials and nerve conduction velocity, were described (Araki et al., 2000; Lille et al,. 1988). In animal models, lead treatment induced EEG disorders and learning disability in young rats (Kumar and Desiraju, 1992). In earlier studies of the Laboratory, similar changes were found in rats after up to 12 weeks oral exposure by Pb^{2+} (Nagymajtényi et al., 1997, 2000).

The absorption of Pb^{2+} and its distribution within the organism is interconnected with that of Ca^{2+} at several points due to chemical similarity. This also results in interference with a number of regulatory processes. Pb^{2+} interferes with Ca-dependent regulation of protein kinase C, calmodulin, ATPases, etc. (Sandhir & Gill, 1993; Bettaiya et al., 1996). Partly due to the interference with Ca^{2+}, lead also affects several transmitter systems. GABA uptake was decreased and dopamine uptake increased in synaptosomes from lead-treated rat brains (Jablonska et al., 1994). Alterations in the dopaminergic, cholinergic and glutamatergic control of behaviour were also observed in lead-treated animals (Cory-Schlechta, 1995; Minnema and Micheaelson, 1986; Minnema et. al., 1986,1988).

1.5. The toxicity of manganese

In contrast to lead and mercury, manganese is essential for living organisms in small amounts, and is toxic only when overdosed (WHO, 1981). Manganese is necessary, among others, for the development and functioning of the brain (Sloot and Gramsbergen, 1994). For man, the main source of manganese supply is food, and to a lesser extent, drinking water. In piped drinking water, Mn levels are kept low (much below the health limit) to avoid brown stains and off-taste. Under certain geological circumstances, however, ground water can have unusually high Mn concentration resulting in toxic exposure of people drinking that water.

Except for that, exposure to abnormally high levels of manganese has traditionally been an occupational risk factor in, e.g., mining and the metal industry. Today, elevated Mn levels are found in the general environment, largely due to human activity. In several countries, the use of the organometal manganese compound methylcyclopentadienyl manganese tricarbonyl (MMT) as anti-knock petrol additive (instead of tetraethyl lead) is a source of air pollution by Mn (Davis et al., 1999; Lynam et al., 1999). Other Mn compounds have widespread agricultural application as fungicides (Ferraz et al., 1988). Spent dry cells, discarded with the household waste, increase the Mn load of dumpsites (ATSDR, 2000). There is a fundamental difference in the state of the global environment in terms of the three metals involved in the present investigation. Lead and mercury emissions were huge throughout nearly the whole 20th century so that the environmental levels – and the corresponding human exposure – are still considerable in spite of the measures applied recently. In case of manganese, the situation is not yet alarming, and the lessons learned from consequences of leaded petrol, together with the toxicological knowledge achieved by now, provide a firm base of avoiding another global pollution problem.

In humans, the lungs and the brain are the organs most affected in chronic Mn exposure. Manganism, the chronic disease resulting from long-term, high-dose occupational exposure to manganese-containing dusts and fumes, has some central nervous system manifestations. The syndrome appearing in manganism, dominated by motor abnormalities (slowed motion, increased muscle tone), resembles Parkinson's disease and is referred to as "drug-induced Parkinsonism" (Montastruc et al., 1994). In the affected persons, functional (Shinotoh et al., 1997) and structural (Yamada et al., 1986) damages to the dopaminergic systems were found. In animal studies, exposure to manganese decreased brain dopamine levels (Bonilla and Diaz-Ewald, 1974; Parenti et al., 1988). Tyrosine hydroxylation, a crucial step of dopamine synthesis, was blocked by Mn in vitro (Hirata et al., 2001) possibly by a mechanism depending on inhibition of mitochondrial function (Zhang et al., 2003). Manganese was found to interfere with synaptic functions in the CNS of experimental animals in several ways. Mn^{2+} is known to block voltage-dependent Ca-channels of neurons and presynaptic endings (Nelson, 1986). Calcium currents of cortical neurons, induced by application of excitatory amino acids, were blocked by Mn^{2+} (Pumain et al., 1987). The release of transmitters, both excitatory (glutamate) and inhibitory (GABA) was reduced by moderate doses of Mn^{2+} (Takeda et al., 2003). Transmitter uptake, an important deactivation mechanism, was also affected by Mn^{2+}. Inhibition of glutamate uptake by cultured astrocytes was reported by Hazell and Norenberg, 1997.

Alterations in the movement of Ca^{2+}, in mitochondrial energy production and in the release and uptake of transmitters are likely to influence the electrical activity of the CNS. In spite of this, literature on Mn effects on spontaneous or evoked cortical activity is minimal. Only a few authors found neurological (EEG and/or evoked potential) disturbances following occupational (Sinczuk-Walczak et al., 2001; Sjögren et al., 1996) or accidental (Hernandez et al., 2003) Mn exposure.

1.6 Combined exposures involving heavy metals in the population and in experimental settings

In experimental hygienic toxicology, the adequacy of work in animals to model human exposure and its consequences has always been a question of debate (Dayan, 2000; Lotti, 2000). Beyond basic species differences, and doses or ways of exposure being not always an exact model of what happens to humans, a major source of limited adequacy is that most of the experimental work is done with a single chemical. According to an estimation, approximately 95% of the resources in toxicology are still devoted to studies of single chemicals (Yang, 1994) which is true also for the investigation of metals (Beyersmann, 1994; Madden and Fowler, 2000). Real life exposure, on the contrary, is rarely limited to a single toxic substance which results in a great variety of potential, and actual, interactions.

Concerning absorption (the link between external and internal exposure), metals in food and drinking water, if conveyed by the same intestinal transporter, can be in competition. Intestinal absorption of lead, for example, is influenced by the calcium demand of the organism and the calcium supply in the food. In case of manganese, absorption in the intestine and passage through the blood-brain barrier goes in competition with iron (Aschner et al., 1999).

Two further common points in the metabolism and effect of heavy metals, induction of metallothioneins and transport or removal by means of these, and interference with Ca^{2+}-dependent phenomena, were mentioned above (1.2).

Combined exposure with several heavy metals is a likely phenomenon in the metallurgy of non-ferrous metals. Several of the ores used to extract such metals are polymetallic, yielding several metals when smelted (and exposing the workers with all of them). Another typical source is production and use of alloys (e.g., manganese, cadmium and nickel is used in steelmaking, or lead, tin, antimony and arsenic in traditional typecasting). Presence of several toxic heavy metals in the environment, and, consequently, in food and water, is frequently seen in areas heavily polluted by industrial activity.

There are several nutritional and lifestyle factors which, if combined with exposure to heavy metals, influence the toxic outcome. Consumption of alcoholic drinks, in varying amounts, is common in the adult population of countries determined by the Euro-American traditions. Ethanol represents the second most widely abused drug in the world after caffeine, and millions of people consume it daily, despite a multitude of problems associated with it. The workforce of smelters and metal-processing plants typically belong to those lower layers of the society (in cultural and economic terms) where excessive use of alcohol is more frequently seen. Certain data in the literature indicate the interaction between alcohol and lead or mercury in case of combined human exposure.

Alcoholism in man is associated with elevated blood lead concentration (Shaper et al., 1982; Dally et al., 1989). In rats, ethanol was shown to enhance the absorption of lead-acetate, to lower the activity of alcohol and aldehyde dehydrogenase (Flora and Tandon, 1987). Moreover, brain tissue levels of noradrenaline were significantly increased and levels of dopamine were decreased by concurrent exposures to lead and ethanol. Ethanol potentiated the toxicity of methylmercury in rats, manifested in neurological signs (hind leg crossing and abnormal gait) and mortality (Tamashiro et al., 1986). In mice, pretreatment with ethanol 0.5 hour before exposure to mercury vapour resulted in markedly lower retention of mercury in blood, brain, heart and lung as compared with the non alcohol-treated controls (Khayat and Shaikh, 1982). Liver mercury content was, however, increased under identical circumstances, with more or less all the hepatocytes apparently engaged in the oxidation of Hg° (Khayat and Dencker, 1983).

Among other mechanisms of damage, ethanol induces oxidative stress in the brain (Sun and Sun, 2001). Presence of certain metals influences the extent of stress. Lead potentiates brain catalase activity and enhances certain effects of alcohol (Correa et al., 2000), and, conversely, ethanol potentiates the lead-induced inhibition of antioxidant defence in rat brain (Jindal and Gill, 1999). Manganese, having several oxidation states between +2 and +7, is engaged both in generation of reactive oxygen species and in protection against oxidative damage by breaking down superoxide anions (by means of the Mn-superoxide dismutase enzyme) and counteracting the oxidative effect of other toxic metals, e.g. mercury (Verity, 1999).

At the Laboratory, the neurotoxic effects of environmental pollutant heavy metals have been investigated for about 10 years. Previous works were usually done with exposure times from 24 hours to 12 weeks and with a single metal. In experimental hygienic toxicology, however, the final goal of research is to provide better protection for human populations, and as outlined above, one-substance exposure is usually not a really good model

of what is experienced by humans. It was thus decided to investigate the effects of combinations of toxicants, frequently encountered by members of the population. One of such neurotoxicants is alcohol. In a previous, subchronic experiment, lead and mercury was given alone or in combination with 5% ethanol in the drinking water (Nagymajtényi et al., 2000). The results showed that the combined application of the above neurotoxic substances induced more marked alterations in the investigated neurophysiological parameters, than if they were given alone. Metal-metal combinations, with metals worked upon in this study and with others, were also investigated in subchronic exposure and some interactions were found (Nagymajtényi et al., 2003).

1.7. Aims of the study

In this study, the experiments performed with acute intraperitoneal treatment with the environmental neurotoxicant heavy metals lead, mercury and manganese, and their combinations with each other and with alcohol, are described. By opting for acute treatment, it was hoped that new information on alterations in the central and peripheral nervous activity could be obtained in a cost- and time-efficient way. Further it was a new dosing and recording regime, completing those used before in the Laboratory to study the effects of heavy metals. It was also supposed that by simultaneous recording several types of nervous activity and describing the alterations on application of the metals and combinations, and by detecting correlation between two activity forms, an insight into the involved mechanisms could be obtained. It was finally supposed that the outcome of these experiments would provide the basis of further studies with a possible application in finding more adequate indicators of effects of heavy metals on the human nervous system.

2. Materials and Methods

2.1. The somatosensory system of the rat as experimental object

In the experiments outlined below, two parts of the somatosensory system of the rat were used as study object.

The majority of the records were obtained using the sensory pathway connecting the whiskers and their projection area in the sensory cortex. The whiskers (vibrissae) are important tactile organs for the rats (wild rats live usually in dark and narrow places). The follicles of the whiskers have various sensory endings, innervated by the trigeminal nerve. This projects to the trigeminal complex in the brainstem, which sends projections to the medial ventral posterior nucleus of the thalamus (VPM). Finally, the thalamocortical projections end in the "barrel field", a region of the somatosensory cortex named after its peculiar cytoarchitecture (Woolsey and Van der Loos, 1970).

The afferent fibres of the tail nerve (more precisely, there is a pair of dorsolateral and ventrolateral nerves) enter the spinal cord in the 3rd sacral to 3rd coccygeal segments. The proximal axon immediately turns up the cord towards the brain and synapses in the medulla. The secondary afferents cross immediately, and in thalamus, they will synapse, and a final neuron will go to somatosensory cortical area. Fig. 1 shows a simple scheme of the pathways involved in the experiments.

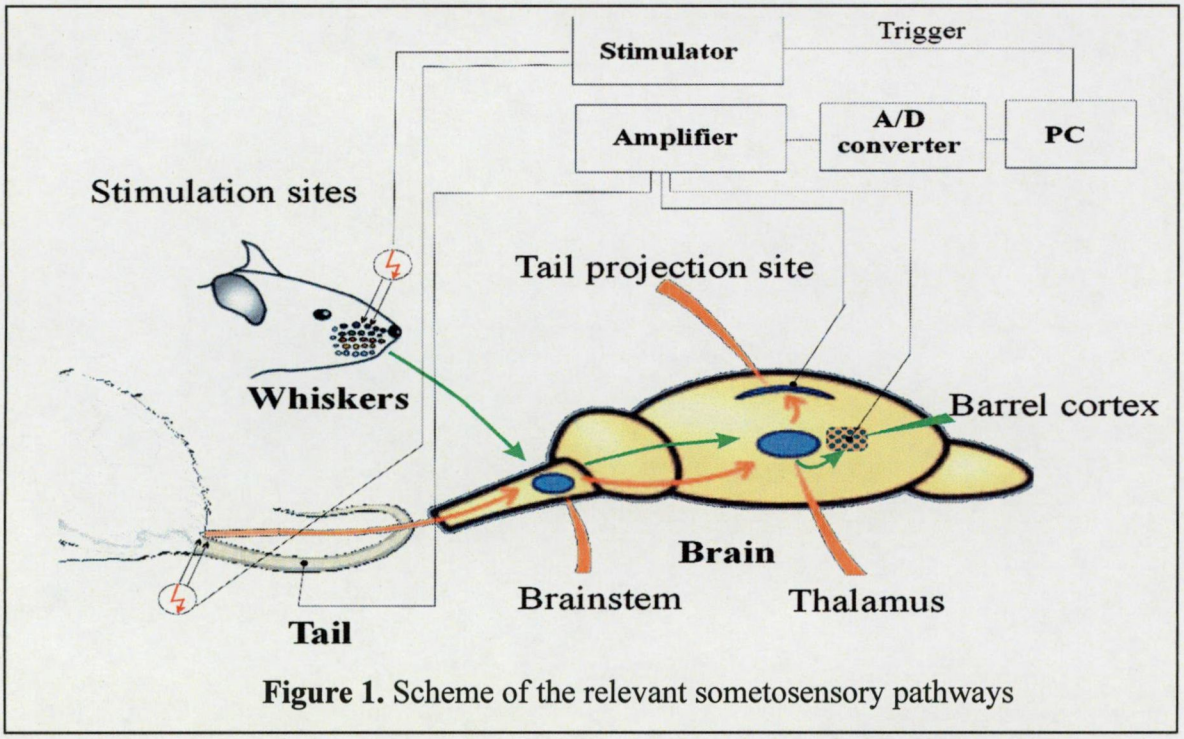

Figure 1. Scheme of the relevant sometosensory pathways

2.2 Preparation of the rats

Adult male Wistar rats (ca. 350 g body weight) were used for the experiments. The animals were purchased at the University's breeding plant and kept under standard conditions until used. Alcohol-pretreated rats had 5% ethanol in their drinking water for 4 weeks, resulting in a daily dose of 4-5 g/kg b.w. alcohol (Olfert et al., 1993). Rats without pretreatment had normal tapwater.

For preparation, the animals were anaesthetised with ip. urethane (1000 mg/kg b.w.; Bowman and Rand, 1980). The head was fixed in a stereotaxic frame and the left hemisphere was exposed by a mid-sagittal cut through the head skin, blunt removal of the muscles and connective tissue attached to the skull, and opening the skull above the left parietal lobe by means of a mini drill. Wound edges were sprayed with 10% lidocaine and the exposed cortex was covered with warm paraffin oil. After an hour of recovery, the rat was transferred into the stereotaxic frame of the recording setup which had a thermostated (+36.5 °C) base plate to maintain the animal's normal body temperature during the session. Ball-tipped silver recording electrodes were positioned on the somatosensory projection area of the contralateral whisker pad (barrel field) and of the tail. These sites were identified with the aid of a somatotopic map (Tracey and Waite, 1995). A stainless steel clamp served as indifferent electrode, attached to the cut skin. In a few animals (5 per treatment group) the thalamic (VPM) evoked response on whisker stimulation was also recorded by a tungsten needle electrode (WPI TM33B10) inserted to the corresponding stereotaxic coordinates (Paxinos and Watson, 1982). A bipolar stimulating needle electrode was inserted in the contralateral whisker pad. In the tail, a pair of stimulating needle electrodes was placed at the base of tail and another pair was placed 50 mm distally for recording. The biological signals to be recorded were amplified (10^4x) and fed into the digitizer interface of the recording setup (see below).

2.3 Recording procedure and acute treatment

The pattern of recording, repeated every 20 minutes, consisted of a five minutes electrocorticogram (ECoG) taken from both projection site simultaneously, followed by evoked potentials (EPs) recorded by applying one train of 20 stimuli to the whiskers and then to the tail. Square pulses (2-4 V, 0.05 ms, 1 Hz) were applied, delivered by a digital time base and stimulator unit (Experimetria, U.K.). The stimulus strength was set to just-supramaximal (that is, the voltage was increased until the evoked response reached maximal amplitude and ca. 5% was added). During stimulation of the tail, compound action potentials of the tail nerve

were also recorded and processed as above. A standard PC and the software NEUROSYS 1.11 (Experimetria, U.K.) was used for recording and evaluation.

After 4 to 5 pre-administration control records, i.e., when the state of the animal was sufficiently stable, one of the heavy metals or a combination was administered by ip. injection (for doses, see Table 1). Parallel control rats received an injection of distilled water. The recording was continued with unchanged pattern until at least 8 further records (over a time span of 160 minutes after administration) were taken. At this time, the rat was over the 4th hour of anaesthesia already (1 hour recovery after surgery, at least 1 hour of control records, plus the time after metal administration) and it was usually found that general state of the animals started to deteriorate after ca. 5 hours. This set a time limit for the length of the session at the end of which the rats were sacrificed by an overdose of urethane. Each dose or combination was given to 8 rats.

Table 1. Doses of the heavy metals in single application

Metal	**Chemical form**	**Dose (pure metal) mg/kg b.w.**	
Lead	$Pb(CH_3COO)_2$	high	1000
		low	500
Mercury	$HgCl_2$	high	7.0
		low	3.5
Manganese	$MnCl_2$	high	50
		low	25
Control (parallel)	Distilled water	--	

The metals compounds were dissolved in distilled water to 1 ml/kg administration volume.

The doses were determined in preliminary experiments, based on previous results of the laboratory (Nagymajtényi et al., 1997; Schulz et al., 1997). The higher dose was set so as to give a firm effect without much general acute toxicity within the above mentioned time limit. Previous experience showed also that the 4 weeks alcohol pretreatment alone had no major effect on the examined parameters (Nagymajtényi et al., 2000). Consequently, no "alcohol only" group had to be involved.

The neurotoxic effects of the investigated heavy metals in a combined exposure situation were studied in two ways: metal-metal combinations were administered, and the metals were given to rats pretreated with alcohol. Although the lower dose of each metal was used in all combination treatments to avoid massive toxicity, one of the possible combinations could not be realized (Table 2).

Table 2. Metal-metal combination treatments

Combinations		Doses (pure metal) mg/kg	
Lead + Mercury	Lead	low	500
	Mercury	low	3.5
Lead + Manganese	Lead	low	500
	Manganese	low	25
Manganese + Mercury *	Manganese	low	25
	Mercury	low	3.5

*These animals died in ten minutes after administration of the mercury and manganese in this combination.

For the metal-alcohol combination, alcohol-pretreated rats (see above) were taken and the low dose of the metals was administered as described.

During the whole procedure, the guidelines by the Ethical Committee for the Protection of Animals in Research of the University of Szeged were strictly followed.

2.4 Measurements and evaluation of the records

From the ECoG records, band activity (standard, delta to gamma; Kandel and Schwartz, 1985) was automatically determined by the evaluation routine of the software, the result being a table of relative band-by-band distribution of the total ECoG power. From that, the so-called ECoG index (Dési, 1983) was calculated (relation of the low and high frequencies in the recorded ECoG; [delta+theta]/[$beta_1$+$beta_2$]) which expresses alterations of the ECoG in a single numeric variable. The evoked activity records - cortical (and thalamic) EPs and tail nerve potential - were automatically averaged. On the EPs, latency and amplitude of the principal wave measured manually, by means of screen cursors of the software (Fig. 2A). Latency of the two main peaks of the EP obtained by whisker stimulation was separately measured but the alterations of the two were parallel, so only the latency of the 1st peak, and the effects on that, was considered. The EP obtained by stimulation of the tail was not sharp enough for an accurate peak latency measurement.

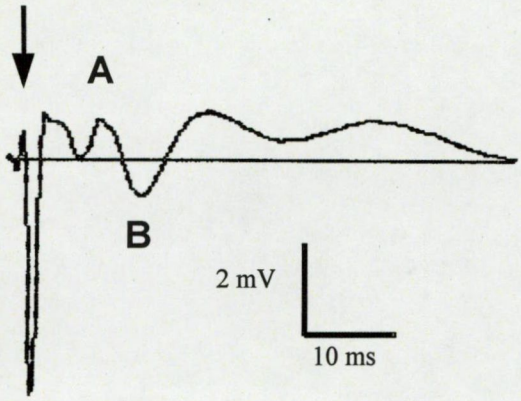

Figure 2A. Original record of the cortical response evoked by whisker stimulation. Amplitude was measured between the two main peaks (***A*** and ***B***) and latencies, between the stimulus artefact (arrow) and ***A*** or ***B***.

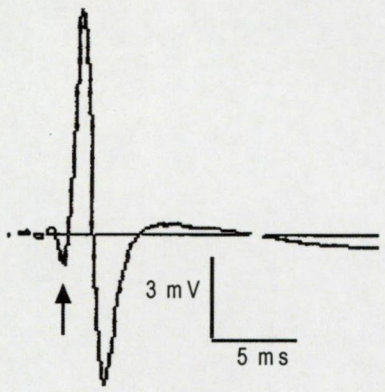

Figure 2B. Original record of tail nerve action potential evoked by tail stimulation.
Amplitude was measured peak-to-peak (as in 2A), and onset latency, between the stimulus artefact (at 2 ms) and onset of the first peak (arrow).

On the compound action potential of the tail nerve, peak-to-peak amplitude and onset latency (Fig. 2B) was measured after averaging. From the latter, conduction velocity was calculated, based on the known distance between the site of stimulation and recording (see above).

2.5 Data analysis and statistics

To eliminate animal-to-animal differences, relative changes were calculated. To do that, the average of the 4 or 5 pre-administration measurements was taken as basis, and all individual measurements (including the control values themselves) were expressed in proportions of that. This calculation was done on all parameters (ECoG index, amplitudes and latencies, conduction velocity) and then the relative values were averaged group by group.

As the effects of the treatment applied (metal or combination) appeared and evolved during the recording session, the most important information was provided by the time trends. These were analyzed by comparing the trend observed in a treated group to that in the untreated (parallel control) group or another group with different treatment.

The significance of the trend difference between a given parameter (ECoG index etc.) from two groups with different treatment, the "general linear model" of the SPSS statistical

software package (SPSS, Chicago, USA) was applied which uses univariate ANOVA to test regression. Group was taken as fixed variable, time as covariate, and the significance of *group and time* effect was tested. Post hoc analysis was performed by LSD, $p<0.05$ was taken as limit of significance in every case.

A possible correlation of the alterations seen in two different parameters was tested by plotting them against each other. A straight line was fitted on the resulting data points and the correlation coefficient was obtained by EXCEL.

3. Results

3.1. Effects on the spontaneous cortical activity

During the recordings, there was a gradual drift in the cortical activity spectrum of the control animals also, resulting in a detectable increase of the ECoG index (see Methods, 2.3) over time. This possibly indicated a slow deterioration of the anaesthetised animals' general state and was a major reason of limiting the period of recording. Consequently, time course of the ECoG in the treated and control animals was compared in order to see the effect of the metals and combinations on the ECoG.

Among the metals, mercury had the most marked effect on the ECoG. A shift to lower frequencies in the spontaneous cortical activity was observed on the records from the barrel field (Fig. 3A) which appeared soon after administration of the metal and became stronger over time. With the higher mercury dose (7.0 mg/kg Hg^{2+}; Table 1) the change was significant in the 140th minute, but the effect of the low dose was not significant. Lead, in both doses, had the weakest effect on the frequency distribution of the spontaneous cortical activity with the change always remaining below significance. The effect of manganese in the doses given was similar to that of Hg^{2+} but less strong, the trend of shift to lower frequencies failed to reach significance even in the higher dose group.

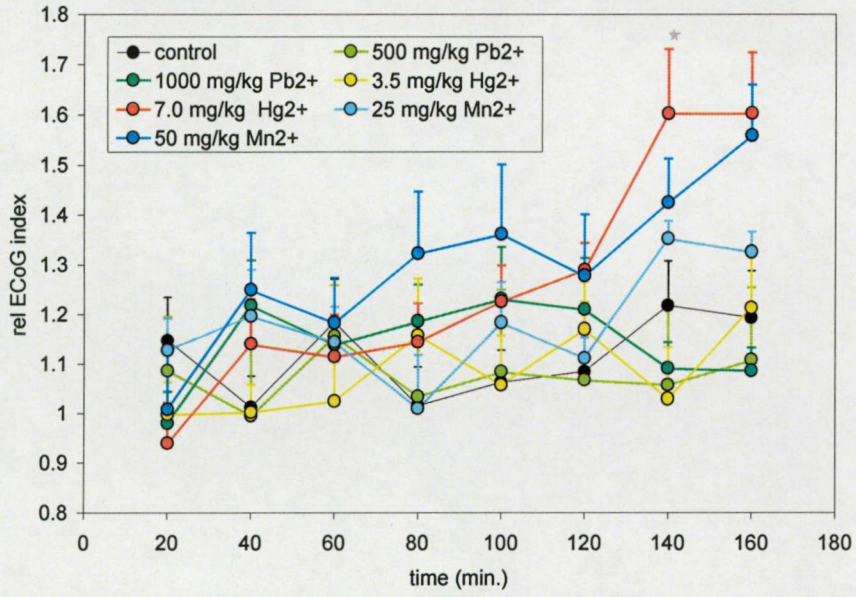

Figure 3A. Effect of the investigated heavy metals on the time trend of the spontaneous activity of the barrel field.
Abscissa: time in 20 min increments from administration of the metal. Data points at the 0th min represent the pre-administration average. Ordinate: change of the ECoG index in relative units, compared to the pre-administration average (given as 0th minute value). The bars represent mean+SEM. ★ = $p<0,05$

The qualitative similarity of the effect of the metals on the spontaneous activity indicated a possible additive (or synergistic) effect. Hence, the lower metal doses (Table 2) were chosen for the metal-metal combinations. Combination with alcohol pretreatment was also done with the lower metal doses, based on literature data and previous experiences (Nagymajtényi et al., 2000).

Compared to the effect of the higher metal doses given alone, the effect of the combinations on the spontaneous activity was of similar direction but weaker (Fig. 3B). None of low dose metals had significant effect in the alcohol-pretreated rats. Similarly, with metals administered in combination (Pb+Mn and Pb+Hg), the effect was below significance.

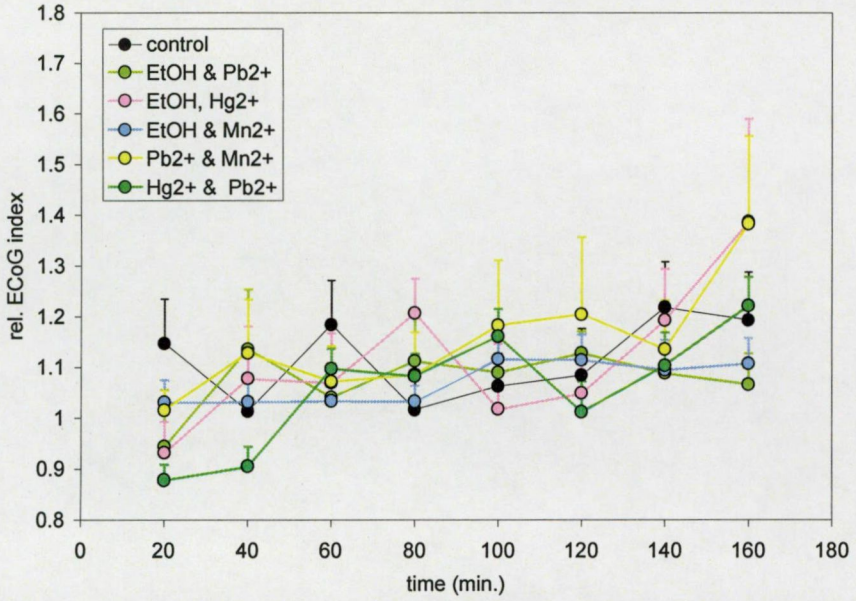

Figure 3B. The effect of the heavy metals on the spontaneous activity in the barrel field in case of metal-alcohol and metal-metal combinations.
Displayed as Fig. 3A.

Locating the cortical projection site of the rat's tail and recording from that was primarily important for observing effects on the evoked activity (see below, 3.4) but provided a chance for observing basal activity in another cortical area beside the barrel field. In the tail projection site, the effects on the ECoG were highly similar to those seen in the barrel field. On ip. administration of 7.0 mg/kg Hg^{2+} the increase of the ECoG index was significant in the 140^{th} and 160^{th} min (Fig. 4A). The increase obtained by high dose Mn^{2+} also reached significance at this recording site (in the 100^{th} min). Similarly to the effects seen in the barrel field, the ECoG index after treatment with Pb^{2+}, with low dose Mn^{2+} or Hg^{2+} (Fig. 4A), with

the metal-metal combinations, or with the low dose metals in rats pretreated with alcohol (Fig. 4B), the change of the ECoG index was not significant.

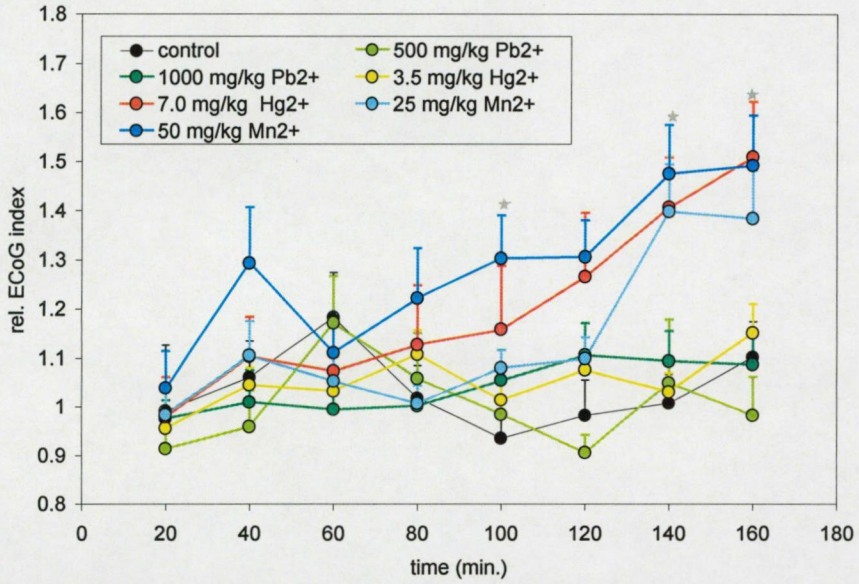

Figure 4A. The effect of the heavy metals on the spontaneous activity recorded from the tail projection site.
Displayed as Fig. 3A.

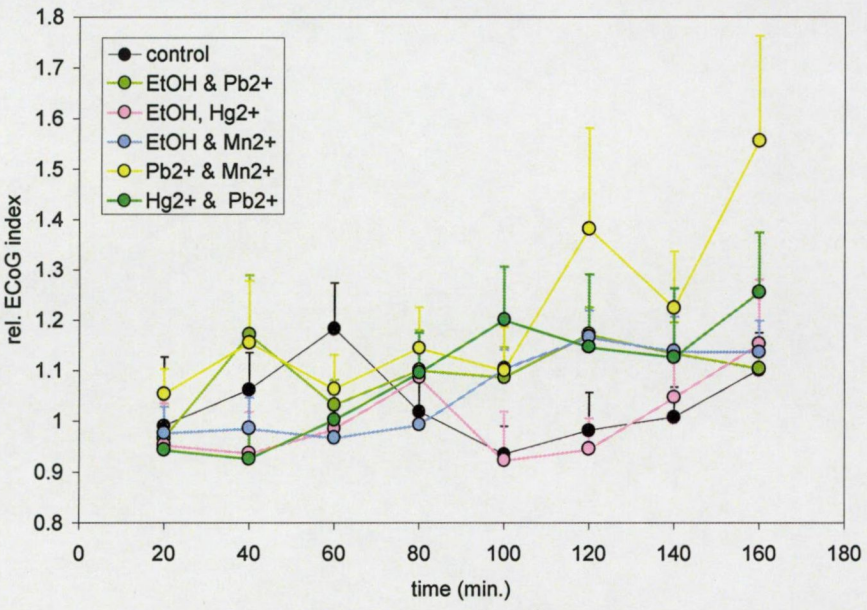

Figure 4B. The effect of the metal-metal and metal-alcohol combinations on the spontaneous activity recorded from the tail projection site.
Displayed as Fig. 3A.

3.2 Effects on the cortical response evoked by stimulation of the whiskers

The parameters of the evoked potentials to be measured and analyzed were amplitude and latency. On these, the effect of the investigated heavy metals was usually more considerable than the effects seen on the spontaneous cortical activity.

The general form of the metal-induced amplitude change was an increase. Similarly to the effects on the ECoG, Hg^{2+} had the strongest effect, followed in this case by Pb^{2+} and Mn^{2+} (Fig. 5A). On ip. dosage of 7.0 mg/kg Hg^{2+}, there was an increase in the response amplitude which started very shortly after administration, developed gradually over time and was significant vs. control in the 100th, 120th and 140th minute. The effect of low dose mercury was not significant. High dose Pb^{2+} also caused an increase of the response amplitude which appeared usually faster than the effect of Hg^{2+} (was significant from the 40th minute on) but was in the final effect less marked. Low dose lead had no significant effect. The amplitude increase obtained by high dose of Mn^{2+} showed a time course similar to that of the effect of Hg^{2+}. The increase itself was, although significant in the 100th and 160th minute, less than in case of the high dose of the two other metals. Low dose manganese had no significant effect.

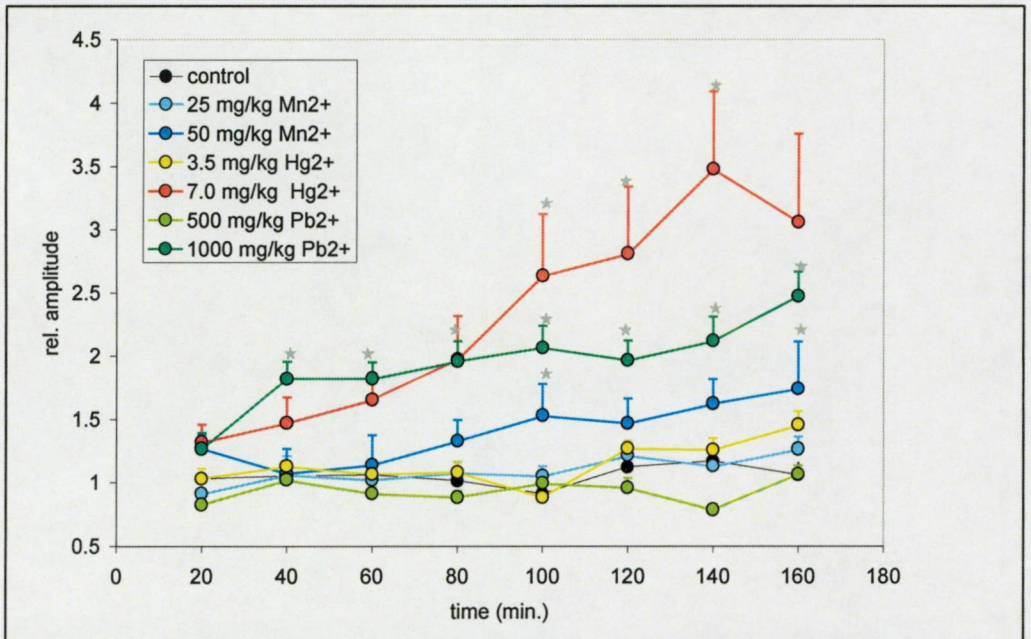

Figure 5A. Amplitude changes of the somatosensory cortical response evoked by whisker stimulation and recorded from the barrel field. Effect of the metals given alone.
Displayed as in Fig. 3A.

The effects of combinations are shown in Fig. 5B. The response amplitude significantly increased on low dose Pb^{2+} in alcohol pretreated rats. The effect of low dose Hg^{2+} and Mn^{2+} combined with alcohol pretreatment was not different from that in non-pretreated rats and was not significant.

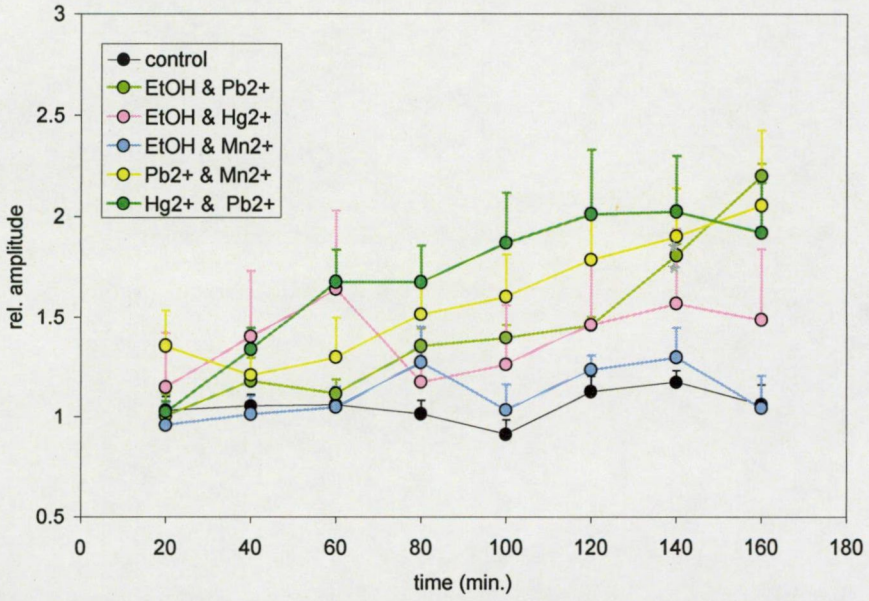

Figure 5B. Amplitude changes of the somatosensory cortical response evoked by whisker stimulation and recorded from the barrel field. Effect of metal-metal and metal-alcohol combinations.
Displayed as in Fig. 3A.

As mentioned before, the time course of the investigated parameters showed a slow, drift-like change mostly also in the untreated (parallel control) rats. Hence, the decisive information about presence or absence of an effect was obtained by comparing the time trends. As seen in Fig. 5C, the effect of low dose Pb^{2+} alone was negligible in non-pretreated rats, but in alcohol-pretreated animals the effect was clearly visible, about half as strong as the effect of the higher lead dose. The interaction of lead with the two other metals in the combinations was also conspicuous. In the Pb^{2+}+Mn^{2+} treated rats, (Fig. 5D) the amplitude increase was stronger than the slight effect of the low dose Pb^{2+} or Mn^{2+} alone, and even stronger than that of the high dose Mn^{2+}. Pb^{2+} and Hg^{2+} also had a positive interaction; the resulting effect was bigger than that of the low dose Pb^{2+} or Hg^{2+} alone but less than that of the high dose Hg^{2+}.

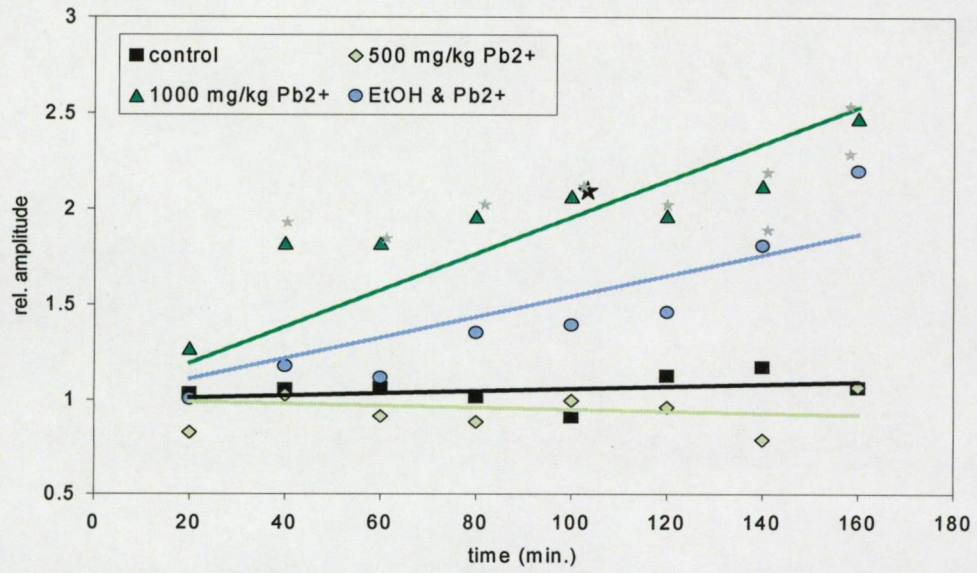

Figure 5C. Amplitude changes of the somatosensory cortical response evoked by whisker stimulation and recorded from the barrel field. Effect of lead, alone and combined with alcohol.
Abscissa: time in 20 min increments from administration. Ordinate: amplitude change in relative units (as in Fig. 3A). Linear trend lines fitted by EXCEL.

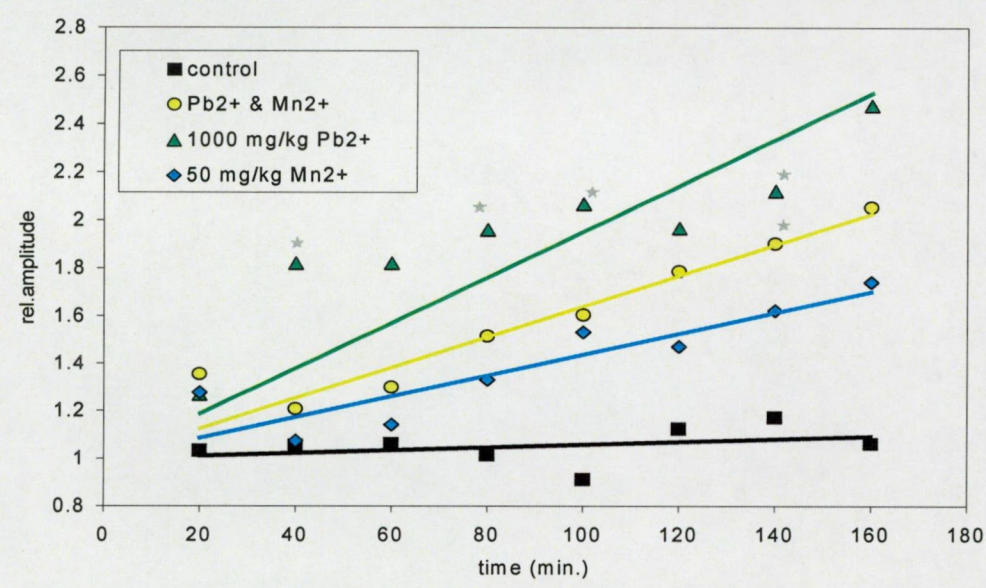

Figure 5D. Amplitude change of the somatosensory cortical response evoked by whisker stimulation. Effect of Pb, Mn and Pb+Mn.
Plotted as Fig. 5C.

Mercury was the only metal which had a massive effect on the latency of the somatosensory evoked potential. The latency lengthening induced by high dose Hg^{2+} evolved with a similar time course as the amplitude increase did (significant from the 40th minute on) while in the animals treated with high dose Pb^{2+} and Mn^{2+} the latency remained at about control level. Similarly, low dose Hg^{2+}, Pb^{2+} and Mn^{2+} caused no significant change (Fig. 6A).

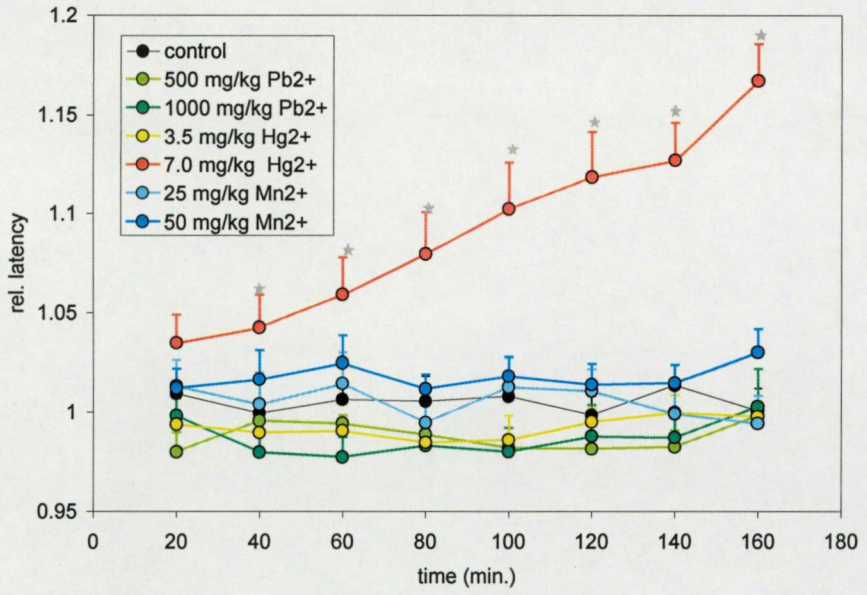

Figure 6A. Latency change of the somatosensory cortical responses evoked by whisker stimulation and recorded from the barrel field. Effects of the metals given alone.
Plotted as in Fig. 5A.

When low dose Hg^{2+} was given to alcohol-pretreated rats, the effect of this combination on the latency of the cortical evoked potential was significant from the 80th minute on (Fig. 6B). Combining low dose Mn^{2+} with alcohol, the increase was significant from the 60th minute. The Pb^{2+}+alcohol combination, however, caused no significant change compared to control or low dose Pb^{2+} alone. The effect of the metal-metal combinations was similar to that seen on the amplitude. Pb+Mn gave a strong potentiation (significant increase from the 60th minute on) while the latency increase obtained by Pb+Hg (significant at the 140th and 160th min) greater than the effect of low dose Hg^{2+} alone but less than the effect of high dose Hg^{2+}.

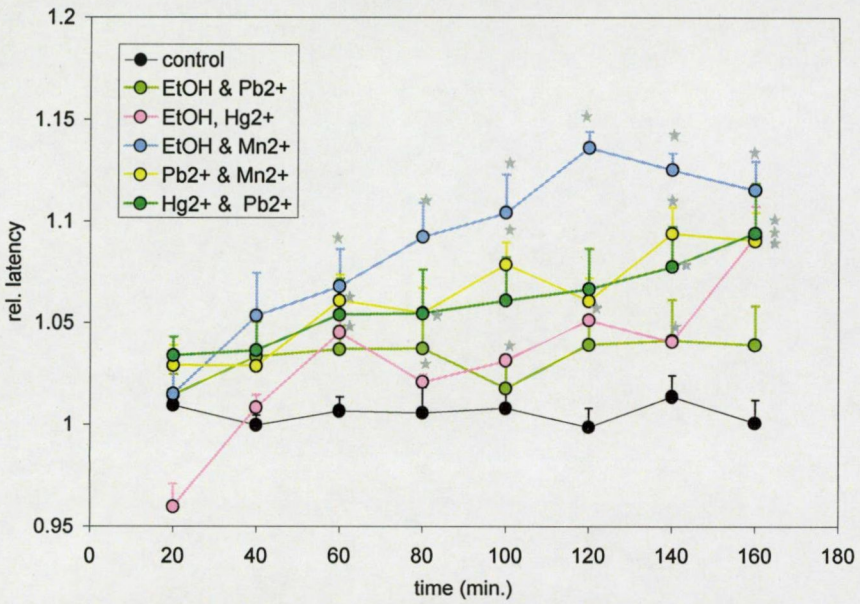

Figure 6B. Latency change of the somatosensory cortical responses evoked by whisker stimulation and recorded from the barrel field. Effects of the metal-metal and metal-alcohol combinations.
Plotted as in Fig. 5A.

In Fig. 6C, the latency lengthening obtained by the two doses of Hg^{2+} in non-pretreated and by low dose Hg^{2+} in alcohol-pretreated rats is visualized by means of trend lines, similarly to Fig. 5C and D.

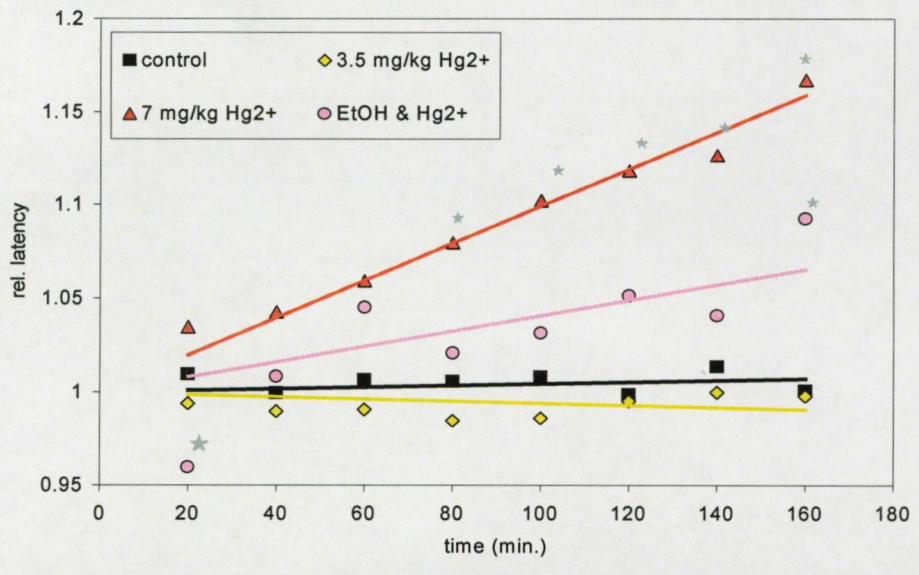

Figure 6C. Latency changes of the somatosensory cortical response evoked by whisker stimulation and recorded from the barrel field. Effect of mercury, alone and combined with alcohol. Plotted as in Fig. 5C.

3.3 Simultaneous recording from the thalamus and the cortex

The effects of the investigated heavy metals on the cortical evoked responses could be induced, theoretically, at any site between the peripheral sensory endings and the cortical projection area. In an attempt to localise the effects, simultaneous recording was performed from the barrel field and the thalamic relay site (VPM nucleus) of the sensory pathway of the whiskers.

As shown by Fig. 7, the effects of the metals on the response amplitude were visible also in the thalamic records. There was no noteworthy difference between the time course of the amplitude changes, and the strength of the effect was mostly also nearly identical. In the time resolution used on Fig. 7, it is better seen than in Fig 5A that there was a metal-specific difference in the evolving of the amplitude change: mercury and manganese caused a transient decrease while in case of lead the increase started nearly immediately. The effects of the metals on the evoked response latency were also nearly identical in the thalamic and cortical recording site.

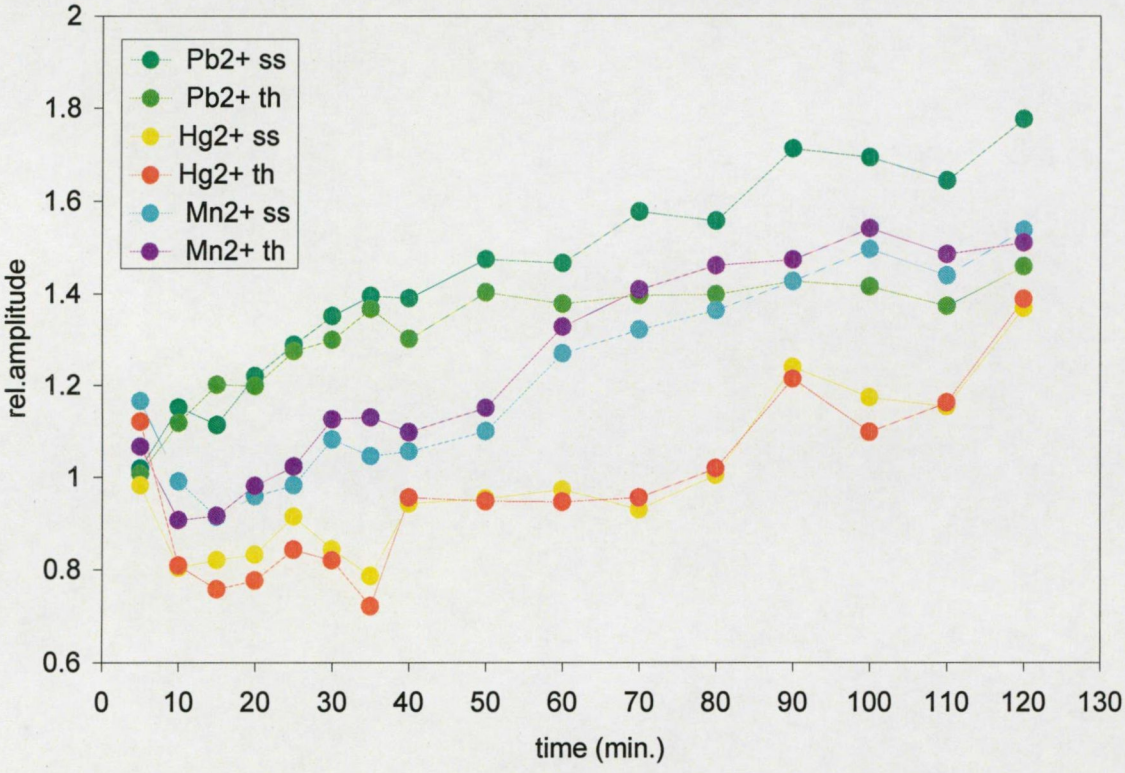

Figure 7. Cortical and thalamic somatosensory responses evoked by whisker stimulation. Effects of high dose of the investigated metals on the response amplitude.
Abscissa: time from the administration of heavy metals. Ordinate: change of the response amplitude in relative units.

3.4 Effects on the cortical response evoked by stimulation of the tail

In the standard recording protocol, used in previous works of the Laboratory, stimulating the rat's tail was only used for recording activity from the tail nerve. It seemed promising, however, to try to find the cortical projection site of the tail, because this way a central and a peripheral response, evoked by the same stimulus, could be recorded. This was another way to localise the effects and to see to what extent a possible action on the peripheral nerve can influence the effect seen in the corresponding cortical focus.

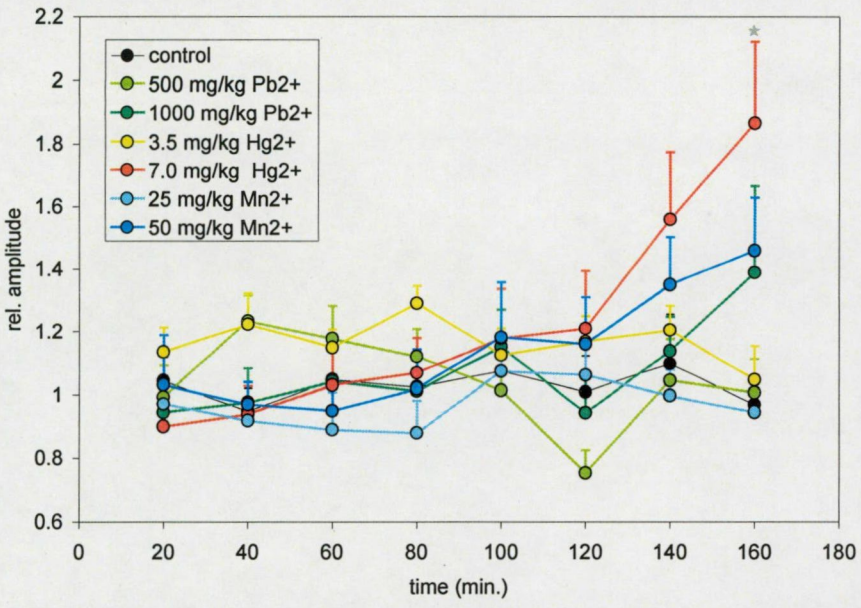

Figure 8A. Amplitude changes of the somatosensory cortical response evoked by tail base stimulation and recorded from the tail projection site. Effect of the metals given alone.
Displayed as in Fig. 5A.

The changes in the cortical response evoked by stimulation of the tail were similar to those observed on the response by whisker stimulation, but less strong. The effects on the response amplitude are shown in Fig. 8A and B. Administration of the high doses of mercury, lead and manganese resulted in an amplitude increase. The effect of Hg^{2+} became significant by the 160^{th} minute. The effect of high dose Pb^{2+} and Mn^{2+} remained below significance, and the low doses of the metals had no noteworthy effect (Fig. 8A).

Among the combinations, an amplitude increase was seen on administration of low dose Mn^{2+} to alcohol-pretreated rats, and with Pb+Mn, but these effects were not significant (Fig. 8B). The other combinations (Pb+EtOH, Hg+EtOH and Pb+Hg) had no effect on the amplitude. The latency time of the cortical response on tail stimulation never showed a

significant change. A slight increase was seen when high dose Mn^{2+}, or Hg^{2+} and Pb^{2+} in combination, was administered.

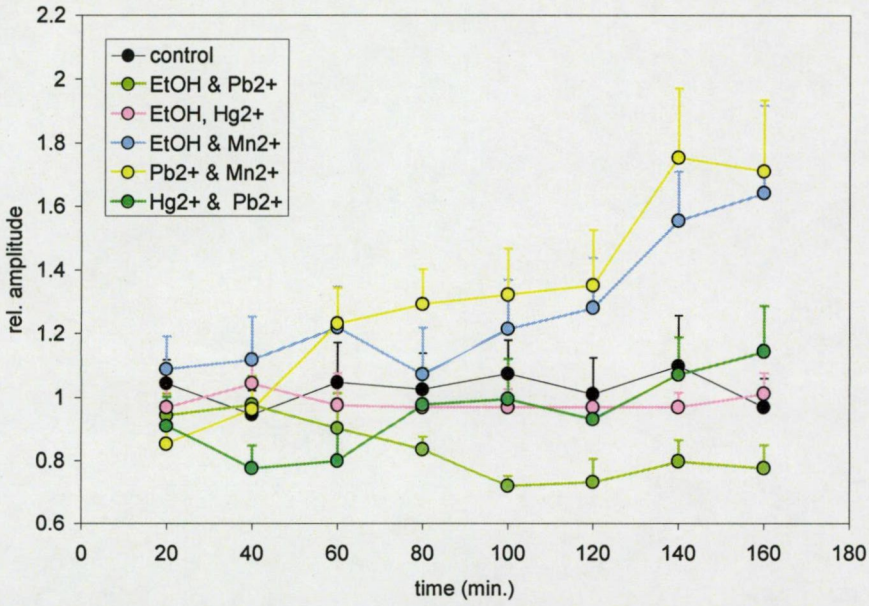

Figure 8B. Amplitude changes of the somatosensory cortical response evoked by tail base stimulation and recorded from the tail projection site. Effect of the combinations.
Plotted as Fig. 5A.

3.5 Effects on the compound action potential of the tail nerve

All three metals caused a gradually evolving, clear decrease of the amplitude of compound action potential of the tail nerve. An important difference against the central nervous effects was that here the low doses of the metals had also an effect when given alone. The general form of the effect was reduction of the tail nerve action potential amplitude, which was the strongest with high dose Hg^{2+}(Fig. 9A). On this parameter, the peculiarity of the Hg^{2+} effect - a transient, weak change in opposite direction to that of the final effect - was a bit more expressed than e.g. on the cortical response amplitude mentioned above. With 7.0 mg/kg Hg^{2+}, a slight, hardly significant increase of the amplitude was seen, followed by gradually evolving clear decrease which was significant from the 120^{th} minute on. The effect of low dose mercury was significant only in the 160^{th} minute. The effect of both doses of lead was also significant. When high dose of manganese administered, the decrease of the amplitude was significant from the 140^{th} minute. Low dose of manganese didn't cause a significant change.

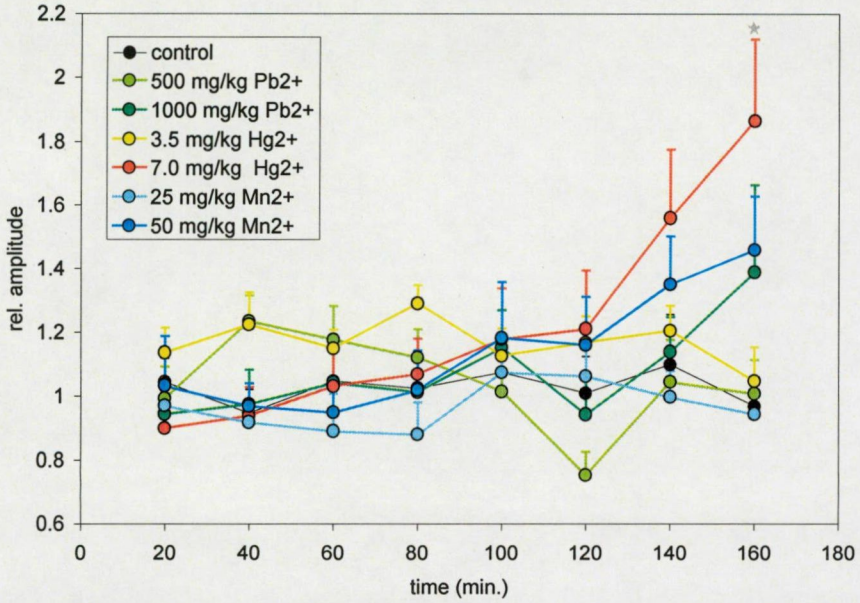

Figure 9A. Amplitude changes of the compound action potential of the tail nerve. Effects of the metals given alone.
Plotted as in Fig. 5A.

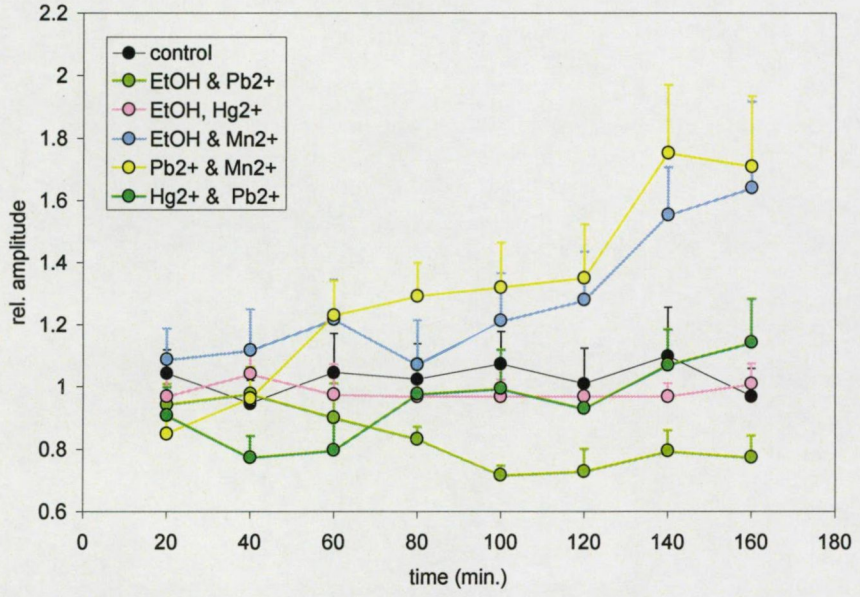

Figure 9B. Amplitude changes of the compound action potential of the tail nerve. Effects of the combinations.
Plotted as in Fig. 5A.

When the low dose of the metals was given to alcohol-pretreated rats, some of the above mentioned alterations were more marked (Fig. 9B). On administration of 3.5 mg/kg Hg^{2+} in the pretreated animals, the amplitude decrease was significant from the 80^{th} minute on. The effect of low dose Mn^{2+} was also stronger (and significant in the 160^{th} minute) in the alcoholised rats.

Among the metal-metal combinations, Pb+Mn gave an effect, which was stronger than the effect of high dose Mn^{2+} or Pb^{2+} alone (significant from the 140th min). The effect of Pb+Hg was not different from that of low dose Hg^{2+}.

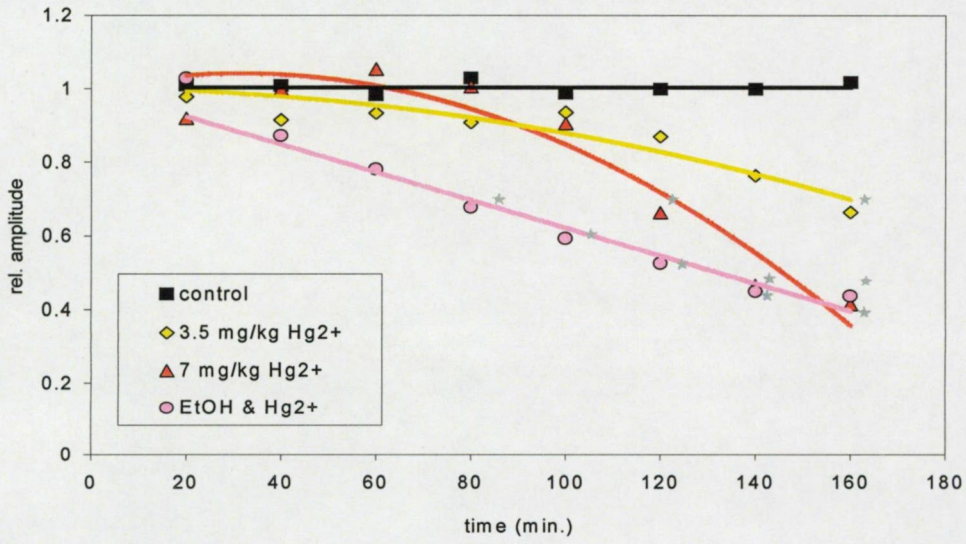

Figure 9C. Amplitude changes of the tail nerve compound action potential. Effect of mercury, alone and combined with alcohol.
Abscissa: time in 20 min increments from administration. Ordinate: amplitude change in relative units (as in Fig. 3A). Trend lines fitted by EXCEL.

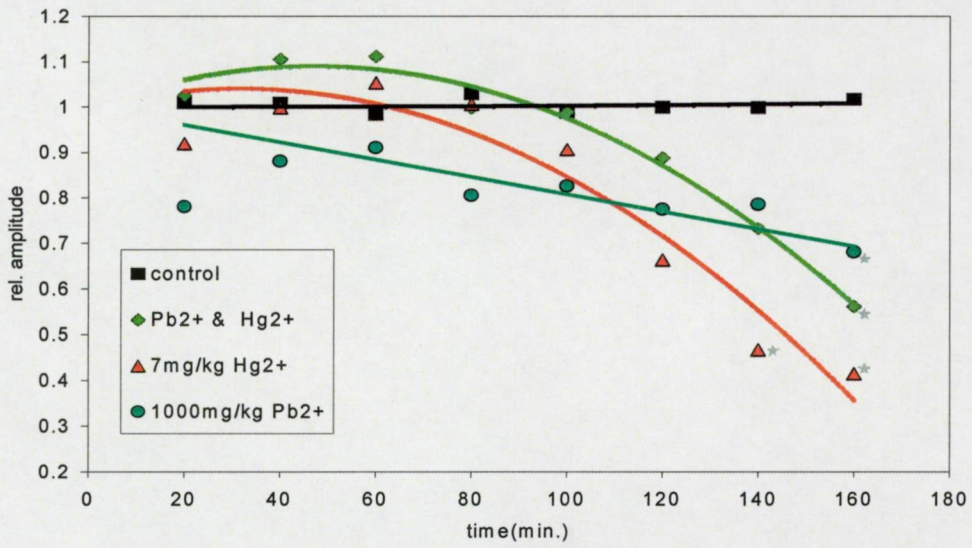

Figure 9D. Amplitude changes of the tail nerve compound action potential. Effect of Hg, Pb and Pb+Hg administration.
Plotted as Fig. 9C.

As in case of the cortical evoked response amplitude, the time trend of the effects on the tail nerve action potential amplitude was visualised on separate plots. As seen in Fig. 9C and D, the trend was markedly different in case of significant effects, although the best fitted line was not straight in all data sets.

In case of the peripheral nerve activity, conduction velocity was the measured temporal parameter. The metals, alone or in the combinations, caused a decrease in the conduction velocity. The decrease after treatment with 7.0 mg/kg Hg^{2+} was significant from the 120th min on (Fig. 10A). With the lower dose, the effect was weaker (significant only in the 160th min). In case of lead, only the effect of the high dose was significant and, as seen in Fig. 10A, it in needed a longer time to develop. On administration of the low dose Pb^{2+}, the effect remained below significance. From the manganese doses, only the higher one altered the conduction velocity (significant reduction from the 100th min on).

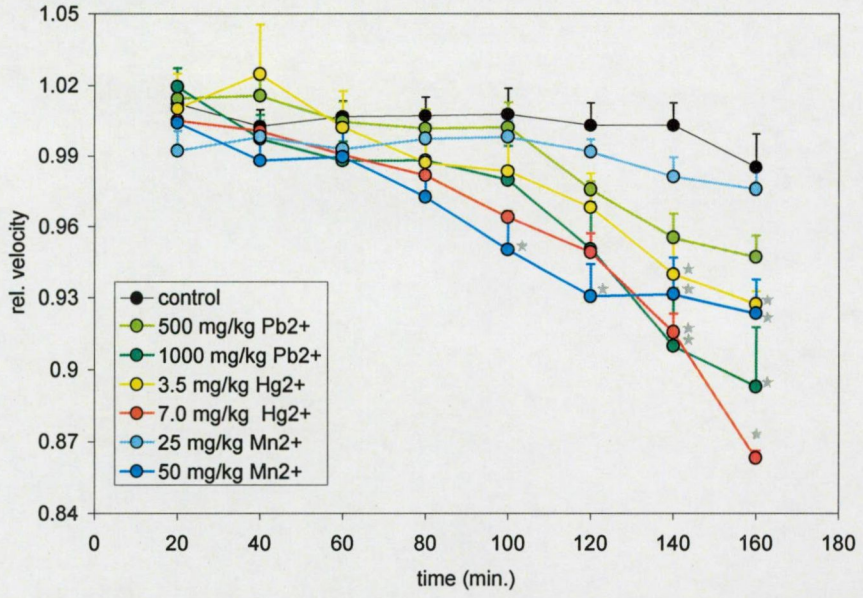

Figure 10A. Conduction velocity of the tail nerve. Effects of the metals given alone.
Plotted as Fig. 3A.

Similarly to what was seen on the tail nerve potential amplitude, the metal-alcohol combinations tended to have a stronger effect than the same metal dose given alone. The decrease of the conduction velocity was significant (120th and 140th minute) when low dose Mn^{2+} was given to alcohol-pretreated rats (Fig. 10B). When low dose Hg^{2+} was combined with alcohol, the effect was only slightly stronger (significant in the 160th min).

When applied in combination, both Pb+Hg and Pb+Mn gave an effect on the conduction velocity which was stronger than the effect of low dose Hg^{2+} or Mn^{2+} alone, and grater (Mn^{2+}) or equal to (Hg^{2+}) that of the high dose metal given alone (Fig. 10B).

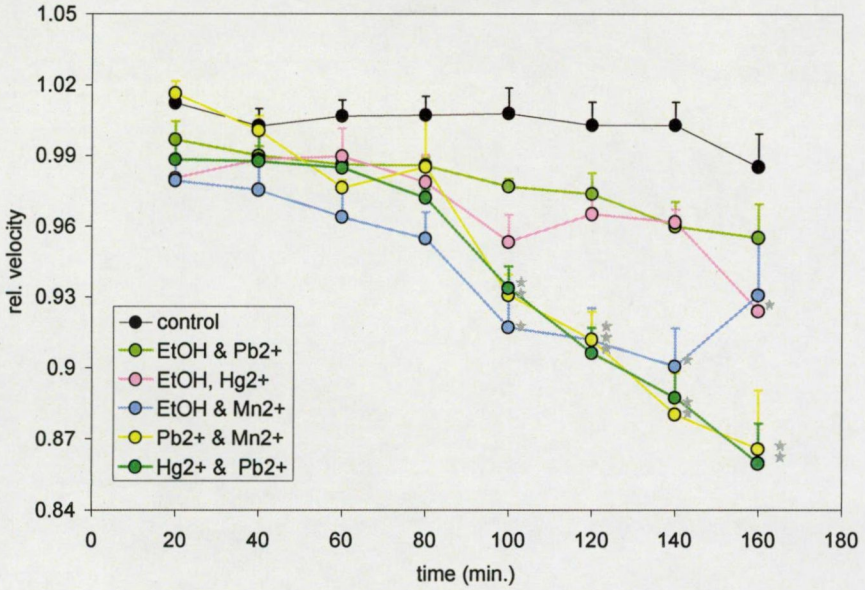

Figure 10B. Conduction velocity of the tail nerve. Effects of the combinations.
Plotted as Fig. 3A.

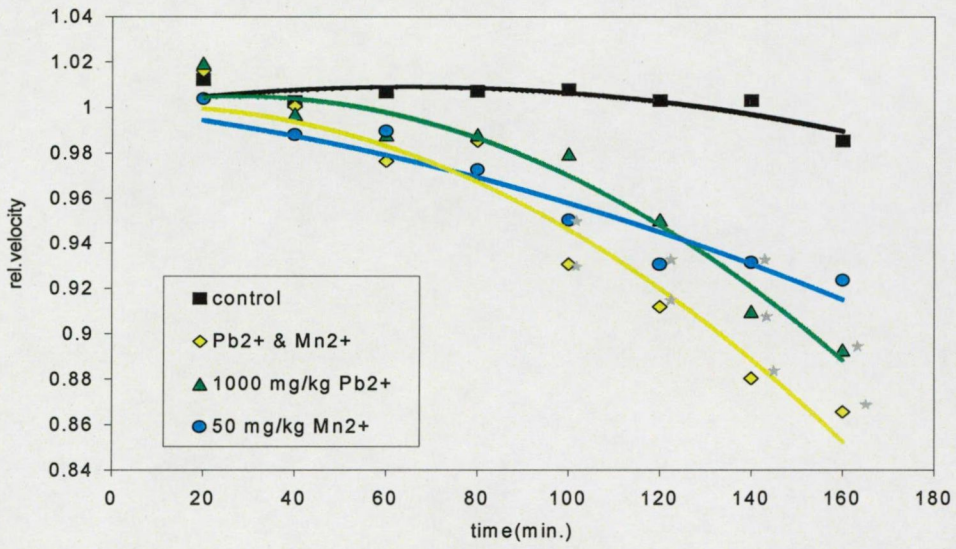

Figure 10C. Conduction velocity of the tail nerve. Effect of lead, manganese and their combination.
Plotted as Fig. 9A.

Fig. 10 C demonstrates by means of trend lines that the time course of the conduction velocity is markedly different in animals treated with lead or manganese, and that the effect of the low dose of these metals, when given together, caused a reduction of the conduction velocity greater than the effect of either metal alone in high dose.

3.6 Correlation of the individual effects

Correlation plots (see Methods) seemed to be a promising means of revealing common mechanisms between the different parameters studied.

In case of the spontaneous and stimulus-evoked activity of the same cortical area, correlation was sought after by plotting the ECoG index (Fig. 3A and B) and the evoked potential amplitude (Fig. 4A and B) in the same graph. As seen in Fig. 11A to D, a fair regression coefficient was obtained for high dose Mn^{2+} and Hg^{2+} (both significant) but a quite poor one for high dose Pb^{2+} (for interpretation, see Discussion). In case of the low doses and combinations, the correlation was poor except for Mn+Pb.

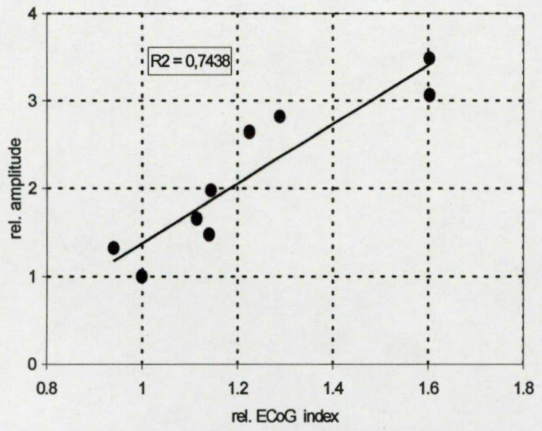

Figure 11A. Correlation plot of the ECoG index vs. evoked potential amplitude. Effect of Hg^{2+}.

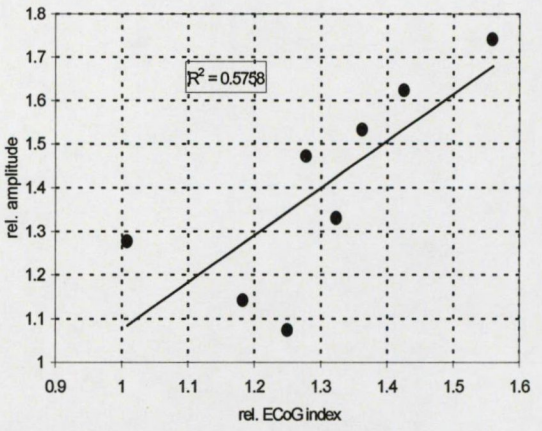

Figure 11B. Correlation plot of the ECoG index vs. evoked potential amplitude. Effect of Mn^{2+}.

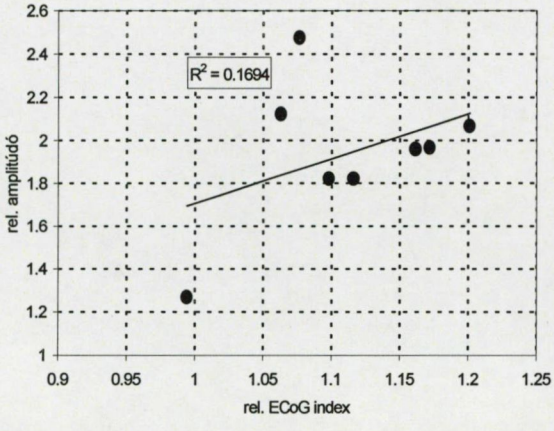

Figure 11C. Correlation plot of the ECoG index vs. evoked potential amplitude. Effect of Pb^{2+}.

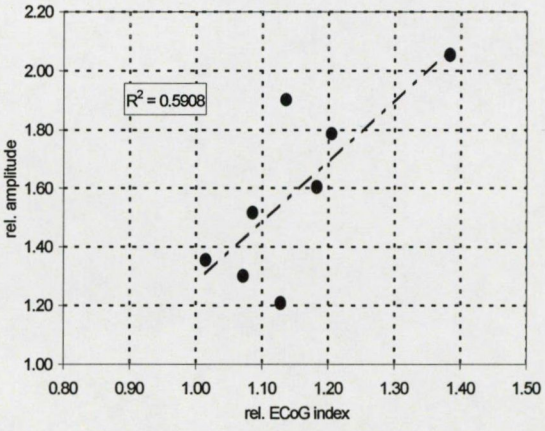

Figure 11D. Correlation plot of the ECoG index vs. evoked potential amplitude. Effect of Mn^{2+}+Hg^{2+} .

As mentioned before, it was supposed that the comparison of alterations in a comparable peripheral and central parameter can give insight into the localisation and/or mechanism of the toxic action. As the latency of the cortical response is necessarily influenced by the speed peripheral excitation arrives into the brain, These two parameters were also put in correlation plots (the tail nerve conduction velocity was plotted together with the cortical response obtained by whisker stimulation, because the evoked response obtained by stimulating the tail was rather flat and had no clear peak latency).

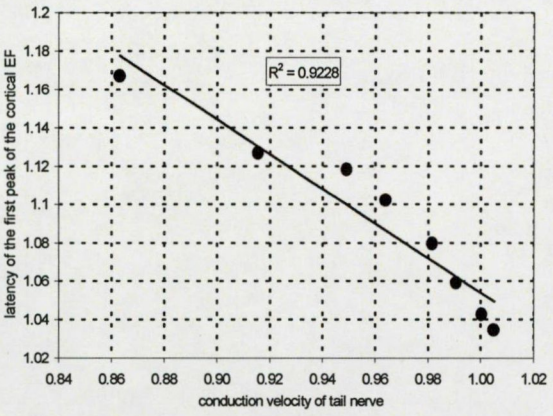

Figure 12. Correlation plot of the tail nerve conduction velocity vs. the cortical response latency. Effect of Hg^{2+}.

In case of high dose Hg^{2+} administration, the correlation of the mentioned parameters was good and significant (Fig. 12). In all other treatment variations, poor correlation was found.

3.7. Summary of the results

In the experiments described above, the effects of acutely administered heavy metals were observed on several types of nervous electrical activity. When the heavy metals lead, mercury and manganese were given alone, in the combinations lead-mercury and lead-manganese, or when giving the metals to alcohol-pretreated rats, changes in the measured or calculated numerical parameters of the cortical spontaneous activity, in the somatosensory cortical evoked potentials, and the tail nerve compound action potential were seen. The effects are summarised in Table 3.

Table 3. Summary of the results with direction and significance of the changes observed.

	EGoG index (barrel field)	ECoG index (tail projection)	cortical EP amplitude (barrel field)	cortical EP latency (barrel field)	cortical EP amplitude (tail projection)	cortical EP latency (tail projection)	tail nerve action potential amplitude	tail nerve conduction velocity
Pb high	∅	∅	↑	∅	↑	∅	↓	↓
Pb low	∅	∅	∅	∅	∅	∅	↓	↓
Hg high	↑	↑	↑	↑	↑	∅	↓	↓
Hg low	∅	∅	∅	∅	∅	∅	↓	↓
Mn high	↑	↑	↑	∅	↑	↑	↓	↓
Mn low	∅	∅	∅	∅	∅	∅	↓	∅
EtOH + Pb	∅	∅	↑	↑	∅	∅	↓	↓
EtOH + Hg	∅	∅	↑	↑	∅	∅	↓	↓
EtOH + Mn	∅	∅	∅	↑	↑	∅	↓	↓
Pb + Mn	∅	∅	↑	↑	↑	↑	↓	↓
Pb + Hg	∅	∅	↑	↑	∅	∅	↓	↓

Arrows: direction of change if noteworthy, red: significant increase, blue: significant decrease ($p < 0.05$ vs. control); ∅: no noteworthy change.

4. Discussion

Among the multitude of substances exposing human day by day - via food, drinking water or air, or in occupational settings - a lot are known or suspected to harm the nervous system. These are of especial interest because the integrity and proper functioning of an individual's central and peripheral nervous system largely determines his or her chances for a successful and productive life which in turn determines the quality of human resources available for the society. In this field, animal experimentation is inevitable in investigating toxic mechanisms and in developing safety information like guidelines or limit values.

In case of experimental exposure to any xenobiotic, the evaluation of the resulting alterations is critically dependent on how effective the administration of the substance was, that is, whether it had sufficient access to the sites of action. In effects on the CNS activity, passage through the blood-brain-barrier is of high importance. Further influencing factors can be the solubility of salts formed by the injected metal ions with anions present in the body fluids (primarily with Cl^-), the formation of metal complexes with small biomolecules, and the binding to macromolecules. As shown by literature data, and evidenced by the time course of the effects seen in our experiments, the metals injected to the rats intraperitoneally were not much hindered in reaching the brain or other parts of the nervous system.

The form of mercury used in the experiments was $HgCl_2$. Inorganic salts of Hg are generally supposed to have low penetration across the blood-brain barrier (Aschner and Aschner 1990). However, following exposure to inorganic mercury compounds, accumulation of mercury within the CNS has been demonstrated (Aposhian et al., 1996) in spite of the tendency of both Hg^+ and Hg^{2+} to bond to plasma proteins. Using up to 10 mg/kg $HgCl_2$ ip. (Möller-Madsen 1990) mercury was found to deposit in cortical and spinal neurons of rats. Following human exposure to inorganic mercury in miners, deposited Hg was found in the post mortem dissected brains (Kosta et al., 1975). An acute dose comparable to what was applied in our experiments (6 mg/kg $HgCl_2$ ip.) was found to damage the blood-brain barrier within 1 hour (Szumanszka et al., 1993) and Hg was deposited in cortical cells in 18 hours (Gajkowska et al., 1992). It is known that mercury in the body can convert between oxidation states of Hg°, Hg^+ and Hg^{2+} (Dunn et al., 1981). Conversion to elementary mercury may have contributed to deposition in the brain.

Lead chloride, the salt of Pb^{2+} likely to be formed after administration, is of low solubility. In spite of that, lead is readily absorbed by various routes of exposure (WHO, 1977). Entering the bloodstream, Pb^{2+} passes the blood-brain barrier above a concentration

threshold (Bradbury and Dean, 1993). In case of high blood lead level (over ca. 4 μM), breakdown of the barrier was observed - possibly as secondary to the Pb^{2+}-induced astrocytic damage and false activation of endothelial protein kinase C. The consequence, brain oedema, was seen both in human victims and experimental animals (Bressler and Goldstein, 1974).

As it was the case of Hg^{2+} and Pb^{2+}, the changes of the cortical activity after ip. injection of Mn^{2+} appeared also rapidly, which suggested that Mn^{2+} was readily absorbed and transported to the CNS (Rabin et al., 1993). Inorganic manganese, once absorbed into the bloodstream, can pass the blood-brain barrier in transferrin-bound form or as free Mn^{2+} ion via a cation transporter (Ashner et al., 1999). Is absorption higher than excretion, Mn tends to deposit in the brain (Mena et al., 1967). In the peripheral nerves, a transport mechanism analogous to that found in the brain was described (Wadhwani et al., 1992).

The data referred to above indicate possible interactions between the investigated metals during absorption and transport. Is, e.g., the blood-brain-barrier rendered "leaky" by Pb^{2+}, the access of not only of Pb^{2+} but also of the co-administered metal to the brain will be increased, resulting in synergistic effect as seen on the cortical evoked potential amplitude (Fig. 5B; the effect of Pb+Hg and Pb+Mn was significantly stronger than that of the metals in single low dose).

Ethanol has been reported to increase the fluidity of nerve cell membranes (Edelfors and Ravn-Jonsen, 1990). On the blood-brain barrier, increased permeability was observed upon acute treatment with ethanol (as well as propanol and buthanol; Gulati et al., 1985). Excessive alcohol intake was indeed associated with high blood lead level in humans (Dally et al., 1989; Shaper et al., 1982).

On the spontaneous cortical activity, the effect of the three metals investigated by us was, when given alone or in the above-mentioned combinations, qualitatively similar: a shift to lower frequencies (Fig. 3A, B; Fig. 4A, B). The strongest effect had Hg^{2+}, followed by Mn^{2+} and Pb^{2+}. In workers chronically exposed to Hg^{0}, slowed EEG was seen (Piikivi and Tolonen, 1989). In lead-exposed children, preponderance of slow wave activity was found (Otto et al., 1985). In manganese-exposed workers, Sinczuk-Walczak et al. (2001) reported generalised and paroxysmal changes on the cortical electrography. One of the primary determinants of the cortical spontaneous activity is ascending cholinergic activation. The basal forebrain cholinergic system, induced by stimulation of the midbrain reticular system (itself receiving collateral input from the sensory pathways) can act as a "final common pathway" to modulate cortical EEG activity (Dringenberg and Vanderwolf. 1996, 1997; Cape

and Jones, 1998). The ascending cholinergic activation was one of the likely sites of action of the heavy metals investigated in this study.

Inorganic mercury can influence this cholinergic activation by inhibiting choline acetyltransferase (Dwivedi et al., 1980) and by decreasing agonist binding on the muscarinic cholinoceptors (Rajanna et al., 1997). Inorganic lead could possibly interfere with the ascending cholinergic activation of the cortex (Metherate et al., 1992) by increasing the spontaneous and decreasing the stimulus-evoked synaptic release of ACh (Suszkiw et al., 1984) - although in our experiments no significant effect of Pb^{2+} on the ECoG was seen. The synergism of Pb^{2+} and Hg^{2+} in our results was thus probably due to fast depletion of the ACh-containing synaptic vesicles because of increased exocytosis and decreased ACh synthesis. For Mn^{2+}, no mechanism of direct effect on the cholinergic activation is known, at least the muscarinic receptors were found not to be influenced by manganese (Villalobos et al., 1994). Here, an action on metabotropic receptors (mGlu2/3) and the resulting negative feedback on synaptic Glu release is what may result in diminished fast EEG activity (Feinberg et al., 2002). In the metal-alcohol combinations, no appreciable interaction was observed in our work. Metal levels in the CNS were most probably increased, compared to rats without alcohol-pretreatment (due to the damaged blood-brain-barrier, see above), but the effect must have been counteracted by alcohol's own effect to increase beta activity in the ECoG. The latter effect was seen on human (Rangaswamy et al., 2002) and animal (Ehlers and Slawecki, 2000) EEG.

Similarly to the effect on the ECoG, the alterations of the cortical EPs induced by acute metal administration were quite alike (order: Hg > Pb > Mn). In this case, however, the effects were more pronounced and interactions were more clear-cut. The increase of the EP amplitude, which was very characteristic, was most probably due to some specific effect – possibly common for the three metals but not identical to any general toxic effect, because then all forms of activity would have been depressed in a more or less uniform manner. The two most likely mechanisms of this specific effect are the influence on the specific thalamocortical afferents and the neurons postsynaptic to them - a direct mechanism; and the well-known relationship between EEG and EPs - an indirect mechanism.

The excitatory input producing the cortical evoked potentials is glutamatergic. Uptake of glutamate by astrocytes, a major factor in terminating its excitatory, and potentially excitotoxic, action (McBean and Roberts, 1985; Robinson et al., 1993) is inhibited by $HgCl_2$ at low doses (Brookes, 1988). This might play a role in central nervous system toxicity of mercury in general (Brookes, 1992; Matyja and Albrecht, 1993), and may explain the increase of evoked potentials under acute Hg^{2+} influence. In rats treated for 12 weeks with lower Hg^{2+} doses (Nagymajtényi et

al., 2000; Papp et al., 2000; Schulz et al., 1997), decreased EP amplitudes were seen, possibly due to the excitotoxicity of excess glutamate for a longer time. The effect of Mn^{2+} is similar but localised one step further in the glutamate metabolism. Following uptake, astrocytes transform glutamate to glutamine by a Mn-dependent enzyme (Hazell and Norenberg, 1997), a process inhibited in astrocytes exposed to elevated Mn^{2+} (Normandin and Hazell, 2002). The influence of lead on transmitter release was demonstrated in vitro in hippocampal neurons, where Pb^{2+} diminished stimulus-evoked release of Glu and GABA (Braga et al., 1999a,b) by a Ca^{2+}-dependent mechanism, similar to what was found by Suszkiw et al. (1984) in rat brain synaptosomes. Supposing a like mechanism in the cortex, the outcome may be more activation and less inhibition of the cortical neurons responsible for the generation of evoked potentials.

Phenomena dependent on or regulated by Ca^{2+} are generally a likely site of action for a number of heavy metals (see above, Introduction 1.2). Mn^{2+} can permeate voltage-dependent Ca^{2+}-channels of cortical neurons but reduces the current through such channels carried by other permeable ions (Nelson, 1986). The same was described for presynaptic Ca^{2+}-channels (Drapeau and Nachshen, 1984). Hg^{2+} and Pb^{2+} are also blockers of voltage-dependent Ca^{2+}-channels (Büsselberg, 1995). This way, each of the metals can slow down the speed of signal propagation along the sensory pathway, resulting in increased cortical EP latency which was observed indeed. The effect of Pb^{2+} and Mn^{2+} is complicated by its effect on transmitter release (Suszkiw et al., 1984; Takeda, 2003).

As shown by the effect of combined exposures, alcohol-pretreatment influenced the metal effect on the EP amplitude only slightly, probably via increased blood-brain-barrier permeability. In the Pb^{2+}+Hg^{2+} and Pb^{2+}+Mn^{2+} combinations, moderate and strong synergism, respectively, was seen (Fig. 5B, C, D; 6B, C; 8B) where the ion channel effect of the metals can be supposed as common point.

The above-mentioned indirect effect on the cortical EP amplitude can be deduced from the effect of the metals and combinations on the ECoG. Under conditions of decreased spontaneous cortical activity - as was seen in our experiments - the amplitude of evoked responses generally increases, as has been described in animals (Herz et al., 1967) and humans (Corletto et al., 1967; Hegerl et al., 1996; Rémond and Lesévre, 1967). As demonstrated in Fig. 11, a fair correlation was found between the alteration of the spontaneous and evoked cortical activity in case of Hg^{2+} and Mn^{2+} treatment. The underlying mechanism is not necessarily the same: For Hg^{2+}, decreased cortical activation may be the primary event, and for Mn^{2+}, increased thalamocortical and decreased intracortical transmission. In case of Mn^{2+} + Pb^{2+}, the only

combination with a good correlation between spontaneous and evoked cortical activity, the common point may be glutamatergic transmission and/or Ca^{2+} channels.

Heavy metals caused, in our investigations, generally an amplitude decrease and a latency increase of the tail nerve action potential, the latter corresponding to a slower conduction velocity (order: $Hg^{2+} > Mn^{2+} \approx Pb^{2+}$; Fig. 10 A). Damage to motor axons by $HgCl_2$ was described by Pamphlett and Coote (1998). The effects of Hg^{2+} on ion channels and Ca^{2+} homeostasis are also known (Denny and Atchison, 1996; Sirois and Atchison, 1996). Lead, acting on voltage-dependent Ca^{2+} and Ca^{2+}-activated K^+-channels, affects ion transport through the neuron membrane (Audesirk and Audesirk, 1991; Reuveney and Narahashi 1991, Leinders and Vijenberg, 1992), thereby slowing down the conduction of action potential and, consequently, increasing the latency of the peripheral nerve action potentials. In case of Mn^{2+}, impairment of mitochondrial energy production (beside the channel effects) can decrease impulse propagation. Mitochondrial complex II (Malecki, 2001) and complex III (Zhang et al., 2003) are inhibited by the presence of Mn^{2+}. Insufficient energy production in the neurons can reduce axonal conduction velocity, first of all in the fast, myelinated fibres producing the major part of the compound peripheral action potential recorded in our study. (In the cortex, reduced energy supply can be another factor leading to the preponderance of slow waves.) Although ethanol is known to slow the rate of membrane depolarisation and decrease conduction velocity of the axon (Stephens, 1992), the effect of metal-alcohol, combinations was, except for Mn^{2+} not significantly different from that of the low dose metal alone. The metal-metal combinations, however, caused a significantly stronger reduction of the conduction velocity than the metals given alone in low dose, indicating a synergism (Fig. 10 B).

In the latency time of the cortical evoked response, all events from the peripheral stimulus till the cortical excitation are summarised. Consequently, at least a fraction of the alterations in the cortical latency must originate from effects on the peripheral sensory nerves. This relationship could be verified, by demonstrating correlation, only in case of Hg^{2+} (Fig. 12).

In hygienic toxicology, the final goal is to improve the health protection of those exposed to toxic chemicals. So it is of interest how the results of the experiments performed in the present study can be utilised for such purposes. Direct transfer of the data from animal experiments to man is seldom correct. The kind and mechanism of interaction between the substances given acutely to rats in our experiments is not yet clear. The results indicate all the same that that combined exposure of humans to the three heavy metals lead, mercury and manganese, and alcohol – which, considering the possible environmental and occupational exposures and

relevant lifestyle factors is not unlikely - may have unexpectedly severe consequences. This points to the need of search for methods capable of early detection of the harmful effects. In further experiments, based on the results presented above but involving longer exposure periods, certain alterations in the activity forms of the central and peripheral nervous system may be identified which react on exposure to the investigated toxicants in a sensitive and specific way. These, in turn, have the potential to be developed to practical biomarkers of human exposure, as it has been suggested in earlier publications form the Laboratory (Dési and Nagymajtényi, 1999; Papp et al., 2000, 2001).

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7. Appendix

1. *Pecze L.*, Papp A.: Parallel changes of the spontaneous and stimulus-evoked cortical activity elicited by acute treatment with inorganic mercury in rats. *Centr. Eur. J. Occup. Environ. Med.* 8;126-130 (2002).
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